

Electron microscopy of aminergic retinal neurons

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This thesis is based on the following papers:

- I. Holmgren, I.: Synaptic organization of the dopaminergic neurons in the retina of the Cynomolgus monkey. Invest. Ophthalmol. & Vis. Sci. 22:8-24, 1982.
- II. Holmgren-Taylor, I.: Ultrastructure and synapses of the (³H)-dopamine-accumulating neurons in the retina of the rabbit. Submitted for publication, 1982.
- III. Ehinger, B. and Holmgren, I.: Electron microscopy of the indoleamine-accumulating neurons in the retina of the rabbit. Cell Tissue Res. 197:175-194, 1979.
- IV. Holmgren-Taylor, I.: Electron microscopical observations on the indoleamine-accumulating neurons and their synaptic connections in the retina of the cat. J. Comp. Neurol. In press, 1982.
- V. Holmgren-Taylor, I.: Synaptic organization of the indoleamine-accumulating neurons in the cyprinid retina. Submitted for publication, 1982.
- VI. Holmgren-Taylor, I.: Synapses of the inner plexiform layer in the retina of cyprinid fish. Submitted for publication, 1982.

The papers will be referred to in the text by their Roman numerals.

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1. INTRODUCTION

There is great variety in the morphology of retinal neurons, both in retinae from one single species and among retinae from different species, and this is seen already on the level of the light microscope. The variability was clearly demonstrated already by Santiago Ramon y Cajal in his systematic investigations of the retinae in a large number of animal species with the Golgi silver impregnation technique. In spite of the capriciousness of this procedure, which literally labels one neuron here and one neuron there, Ramon y Cajal's descriptions were so complete, that it was not until the advent of the electron microscope that the study of the retinal neurons found a way to advance further, and the synaptic connections between neurons could then be investigated. Subsequently cellular structures, which closely resembled those present at the neuromuscular junctions and where chemical transmission of nerve impulses was unequivocally proven physiologically, were seen in the neurons of the central nervous system including the retinal ones. Purely electrical transmission between neurons was also linked to specific cellular specializations on the ultrastructural level.

Early observations on the structure of retinal junctions (Sjöstrand, 1958; Missotten, 1965) and on the cell types in which they appear, were followed in the 1960's by serial section analyses in the electron microscope of the ramification of the neuronal processes and their synaptic connections (Dowling and Boycott, 1966; Raviola and Raviola, 1967). During the past two decades, many fruitful studies have combined serial section analysis in the electron microscope with the Golgi silver impregnation technique or the more selective horseradish peroxidase injection technique with similar methods on the level of the light microscope (see, for example, Stell and Lightfoot, 1975; Kolb, 1979).

Another step towards a more functional morphological classification of retinal neurons and their connections was taken around 1960 when it was discovered that different subpopulations of

retinal neurons could be distinguished on the basis of their pharmacological properties, or, more specifically, on the basis of their presumptive neurotransmitter. There are four main criteria for the identification of a chemical substance as a neurotransmitter:

1. The substance must be synthesized and stored within the neurons, particularly in their presynaptic terminals.
2. Nerve stimulation must lead to the release of the substance from nerve terminals.
3. The actions of the substance on the innervated organ must be the same as the effects of nerve stimulation.
4. There must be mechanisms for the destruction or removal of the proposed transmitter.

In the past twenty years several subpopulations of retinal neurons have been identified in the light microscope through methods based mainly on criteria 1 and 4.

The presence of specific subpopulations of retinal neurons containing catecholamines was demonstrated in several species in the 1960's with the Falck and Hillarp technique for fluorescence microscopy of structures containing biogenic monoamines. Microspectrofluorometry, autoradiography, biochemical and physiological studies have subsequently shown that the catecholamine, which these neurons contain and use as their transmitter, is dopamine (reviews, Ehinger, 1978,1982).

A population of indoleamine-accumulating retinal neurons was also discovered in the fluorescence microscope with the Falck and Hillarp technique after uptake of an exogenous indoleamine (Dowling and Ehinger, 1975; Ehinger and Florén, 1976). Their presence has since been shown in a great number of species (Ehinger and Florén, 1980). Since 5-hydroxytryptamine, serotonin, is a likely neurotransmitter in the brain, the indoleamine-accumulating neurons were first thought to be serotonergic. While this is probably true in the retinae of chicken, goldfish, lizard and frog

(Osborne, Nesselhut, Nicholas and Cuello, 1981; Osborne, Nesselhut, Nicholas, Patel, Cuello and Milstein, 1981; Ehinger 1982), there is much question as to the identity of the transmitter of the indoleamine-accumulating neurons in the mammalian retina (Florén, 1979; Florén and Hansson, 1980; Osborne, 1980; Ehinger, Hansson and Tornqvist, 1981; Ehinger 1982). Nevertheless, the indoleamine-accumulating neurons form distinct subpopulations of amacrine cells in species where they can be demonstrated through their uptake system for exogenous indoleamines, such as 5,6-dihydroxytryptamine and/or tritiated 5-hydroxytryptamine.

Other putative neurotransmitters have in the light microscope been localized to specific populations of retinal neurons (review, Ehinger 1982). Substances such as acetylcholine and the amino acids, gamma-aminobutyric acid and glycine, have been studied mainly through autoradiography of the tritiated compound or its tritiated precursor and, in the case of gamma-aminobutyric acid, also through immunohistochemistry of its synthesizing enzyme. The presence of several small peptides in retinal neurons has also been demonstrated with immunofluorescence techniques. Most of these substances are found in varieties of amacrine cells.

On the level of the electron microscope, it has been more difficult to separate and visualize the pharmacologically different subpopulations. There has been some success with immunocytochemical techniques, where the presence of a presumptive neurotransmitter in a neuron is detected with the help of the antibody to the transmitter or an enzyme in its metabolism (for example, Brandon, Lam, Su and Wu, 1980; Vaughn, Famiglietti, Barber, Saito, Roberts and Ribak, 1981). To date, however, these techniques have been relatively unreliable and usually do not allow good fixation of the material.

There are two other methods, which have been highly successful in visualizing the aminergic neurons in the electron microscope; firstly, a labelling technique using a slightly neurotoxic

transmitter analogue, and secondly, autoradiography using the tritiated neurotransmitter as a labelling substance. These two methods have been used in this investigation to study the dopaminergic and indoleamine-accumulating neurons in the retina of mammals and fish. Because the labelling methods used allow both the identification of the neurons belonging to the specific subpopulations and the study of their synaptic structures, the synaptic contacts of the different neurons could be mapped.

Any theory of retinal function must be based on morphological, biochemical and physiological data, and the elucidation of the synaptic organization of the aminergic retinal neurons, which is the aim of this study, is consequently important in terms of understanding the function of the retina. Further, many investigations have shown considerable variations among species in the morphology, biochemistry and physiology of the retina. It is therefore of added interest to map species differences in the synaptic contacts in order to eventually understand if the variations correspond to significant differences in function. Finally, it is well known that a number of drugs, which are commonly used in clinical practice, influence the uptake and metabolism of dopamine and 5-hydroxytryptamine (see, for example, Ehinger and Florén, 1978 a). In time, it is therefore probable that the knowledge of the synaptic organization of the aminergic retinal neurons will not only aid in understanding the normal and abnormal function of the retina, but also its function under the influence of several therapeutic drugs.

2. MATERIALS AND METHODS

2.1. Animals used

The eyes of young adult rabbits (Oryctolagus cuniculus), cats (Felix domestica) and Cynomolgus monkeys (Macaca fascicularis), were used in the experiments on mammalian eyes. In the experiments on fish eyes, those of carp (Cyprinus carpio) and goldfish (Carassius auratus) of 5-15 cm size were used. All animals were

light adapted and exposed to an ambient laboratory illumination of about 100-200 lux. During the intervals between injections in some experiments, they were kept on a regular night and day light and dark cycle.

2.2. Analysis of synaptic contacts

The methods used in this study to map the synaptic organization of two pharmacologically distinct subpopulations of retinal neurons, the dopaminergic ones and the indoleamine-accumulating ones, are based on two assumptions. The first is that the sites of transmission of information between retinal neurons are characterized by cellular structures, which can be identified in the electron microscope. The neurons in the central nervous system, of which the retina is a part, presumably have both slow and fast ways of influencing each other in terms of function. The slow ways, which would induce trophic changes are not well understood, and no specific cellular structures are known to mediate the trophic signals. For the fast transmission of information, specific sets of structures have been associated with chemical and electrical transmission in the central nervous system, namely synapses and gap junctions (Gray and Guillery, 1966; Palay and Chan-Palay, 1975). At the chemical synapses, vesicles presumed to contain neurotransmitter substances cluster close to the specialized presynaptic cell membrane ready to release their content into the synaptic cleft. The short distance across to the specialized postsynaptic receptors permits transmission times in the millisecond range. Such structures form the basis for the analyses presented in this study. Electrical signal transmission is believed to take place at the gap junctions, but none have been observed involving the dopaminergic or indoleamine-accumulating neurons.

The second basic assumption, on which this study rests, is that the uptake mechanisms responsible for the accumulation of the labelling substances, (^3H)-dopamine and 5,6-dihydroxytryptamine, are specific to and selective for aminergic neurons. There is

little doubt that this is the case for the high affinity uptake systems (Iversen, 1975). As long as reasonable concentrations are used, roughly in the micromolar range or less, there is little risk that other neurons than the aminergic ones are labelled. Such concentrations have been used in the present study. However, the amine uptake systems are not selective enough to distinguish efficiently between various biogenic monoamines, such as noradrenaline, dopamine and indoleamines. If precautions are not taken there is therefore no guarantee that only the endogenous amine is accumulated even when the doses are kept low. Actually, the indoleamine, 5,6-dihydroxytryptamine, will label both dopaminergic and indoleamine-accumulating neurons in the retina, and there is a risk that (^3H)-dopamine will also label both types of neurons. However, as will be shown, this difficulty can be circumvented.

The noradrenaline content in the retina is very low and noradrenergic neurons are not thought to be present in any number that could significantly interfere with the analysis (Osborne, 1981).

2.3. Dopaminergic neurons

In the experiments on the dopaminergic neurons, they were first studied briefly in the fluorescence microscope after treatment according to the Falck and Hillarp technique (Björklund, Falck and Owman, 1972). Because of their dopamine content, they were seen to fluoresce green with the filter settings used. The dopaminergic neurons could also be seen in the fluorescence microscope after the accumulation of the neurotransmitter analogue, 5,6-dihydroxytryptamine, after intravitreal injection. In the Cynomolgus monkey, the eyes were processed for electron microscopy after a single intravitreal 5,6-dihydroxytryptamine injection, but in the case of rabbits, the injection of 5,6-dihydroxytryptamine had to be preceded by treatment with 5,7-dihydroxytryptamine. This was necessary because of the presence of indoleamine-accumulating neurons in the retinae of rabbits (Ehinger and Florén,

1976). The latter cell population will also accumulate 5,6-dihydroxytryptamine, even more avidly than the dopaminergic neurons, and would consequently interfere with the study of the dopaminergic neurons (Ehinger and Florén, 1978 b). However, the repeated treatment with 5,7-dihydroxytryptamine destroyed the processes of the indoleamine-accumulating neurons (Ehinger and Florén, 1978).

The accumulation of 5,6-dihydroxytryptamine in the dopaminergic neurons caused ultrastructural changes in the neurons, mainly in the form of increased density of the membranes of the cell itself and its organelles. The changes were conspicuous in the electron microscope, but did not disturb the normal cytoarchitecture of the cells to any extent that made the cell organelles or the synaptic structures difficult to identify.

The dopaminergic neurons in the retina of the rabbit were also studied with an autoradiographic method. Tritiated dopamine was injected intravitreally after the animal had been treated with a monoamine oxidase inhibitor, Pargyline (R). The (^3H)-dopamine was accumulated in the dopaminergic cells, and the presence of the radioactive isotope was then demonstrated by autoradiography. Control experiments, in which the indoleamine-accumulating neurons were removed with 5,7-dihydroxytryptamine prior to the (^3H)-dopamine injections, showed that the experimental procedure with Pargyline and (^3H)-dopamine only labelled the dopaminergic neurons.

2.4. Indoleamine-accumulating neurons

The indoleamine-accumulating neurons of rabbits, cats and goldfish also accumulated 5,6-dihydroxytryptamine. With the Falck and Hillarp technique, this subpopulation of retinal neurons was seen to fluoresce yellow, but only after the accumulation of the exogenous indoleamine. On the ultrastructural level, 5,6-dihydroxytryptamine caused ultrastructural changes in the indoleamine-accumulating neurons similar to those of the dopaminergic ones. It was therefore possible to study the synaptic organization of the indoleamine-accumulating neurons in the electron microscope with

this labelling technique. The otherwise interfering dopaminergic processes had to be removed first, however, through repeated treatment with 6-hydroxydopamine (Ehinger and Nordenfelt, 1977).

3. RESULT AND DISCUSSION

3.1. Dopaminergic neurons in Cynomolgus monkeys (I)

3.1.1. Distribution of cell bodies and processes

When normal retinae of Cynomolgus monkeys (Old World monkeys) are processed according to the Falck and Hillarp method, green fluorescing cells are seen with most of their cell bodies in the innermost cell row of the inner nuclear layer and with their processes ramifying mainly in the outermost sublayer of the inner plexiform layer. The distribution of greenish-yellow fluorescing cells and of labelled cells is similar when retinae treated with 5,6-dihydroxytryptamine are processed for fluorescence microscopy and electron microscopy respectively. The findings are in complete agreement with those previously made on the dopaminergic neurons in the same monkey (Ehinger, 1966 a; Ehinger and Florén, 1979). Furthermore, the distribution of the dopaminergic cell bodies and processes is that of amacrine cells. More specifically, the dopaminergic neurons in the Cynomolgus monkey retina, resemble the "smaller unistratified amacrine cell" described by Boycott and Dowling, 1969.

A few processes of the dopaminergic neurons are also found to extend into the inner nuclear layer, but not further outwards than the first two cell rows. There is, consequently, no network of dopaminergic fibers in the inner nuclear layer of the Cynomolgus monkey of the kind described for several New World monkeys (Boycott, Dowling, Fischer, Kolb and Laties, 1975). A small number of dopaminergic processes also extend into the inner parts of the inner plexiform layer. Moreover, a small percentage (5-10%) of the dopaminergic cell bodies are situated in the ganglion cell layer and an even smaller number of cell bodies in the

inner plexiform layer. There is no evidence that these dopaminergic cells in the Cynomolgus monkey retina have a distribution of processes or a synaptic organization that is different from those dopaminergic cells which have their cell bodies in the inner nuclear layer. In some instances processes have been seen to extend from a dopaminergic cell body in the ganglion cell layer to the outermost sublayer of the inner plexiform layer and join the network of dopaminergic processes there (Ehinger, 1966 a). Therefore, the majority of the dopaminergic cells found in the inner plexiform and ganglion cell layers, earlier called "eremite" and "aloganglion" cells (Ehinger, 1966 b), are considered to be varieties of amacrine cells. Similar amacrine cells among the ganglion cells have been observed with other methods (Hughes and Vaney, 1980; Masland and Mills, 1979; Hayden, Masland and Mills, 1980).

3.1.2. Labelling with 5,6-dihydroxytryptamine

The ultrastructural changes produced by 5,6-dihydroxytryptamine in the dopaminergic neurons mainly consist of increased density of the cell membranes, especially at synapses and junctions, and of the membranes of cell organelles. There is also distortion of such cell organelles as mitochondria and large dense-cored synaptic vesicles. Small synaptic vesicles often contain an eccentric electron dense spot, and the large dense-cored vesicles have an expanded central core of increased density. Enough of the normal cytoarchitecture remains, however, to allow the unequivocal identification of synapses.

3.1.3. Synaptic contacts

The dopaminergic processes have output synapses of the conventional kind with a presynaptic cluster of small synaptic vesicles, pre- and postsynaptic membrane densities and material in the synaptic cleft. The dopaminergic processes are postsynaptic to processes with amacrine cell characteristics: varicosities alternating with thin intervaricose segments, uneven distribution of

small synaptic vesicles, usually few mitochondria and neurotubules, and conventional output synapses (Dowling and Boycott, 1966). In several instances the dopaminergic processes are seen to make synaptic contact with unlabelled cell bodies in the innermost row of the inner nuclear layer and with the processes descending from a cell body in that first cell row of the inner nuclear layer. Serial and convergent synapses involving dopaminergic processes are also present.

The dopaminergic processes are postsynaptic to processes with amacrine cell characteristics. The number of input synapses observed is only about 5% of the number of output synapses. One reason for this is probably the inconspicuous synaptic structures associated with the input synapses, but even so the difference in numbers appears to validate the conclusion that the sites of synaptic output are more numerous than the sites of synaptic input. Similar differences in the number of output and input synapses detected have been observed for the dopaminergic neurons in the inner plexiform layer of rabbits and Cebus monkeys (Dowling and Ehinger, 1978 a; Holmgren-Taylor, 1982 a; Dowling, Ehinger and Florén, 1980). There are contacts between labelled dopaminergic processes which resemble the conventional type of synapse and also desmosome-like contacts which have symmetrical membrane densities and material in the intercellular space.

3.2. Dopaminergic neurons in rabbits (II)

The dopaminergic neurons in the retina of the rabbit have previously been studied both in the fluorescence microscope with the Falck and Hillarp method and in the electron microscope with the 5,6-dihydroxytryptamine labelling technique (Ehinger 1966 b, c; Ehinger and Falck, 1969; Dowling and Ehinger, 1978 a). On the level of the light microscope, they have also been labelled through their uptake mechanism for (^3H)-dopamine (Ehinger and Falck, 1971; Lam, Fung and Kong, 1981). A similar autoradiographic technique has now been used to study the ultrastructure of the

dopaminergic neurons in the retina of the rabbit. Before the intravitreal injection of (^3H)-dopamine, the experimental animals received an intraperitoneal injection of the monoamine oxidase inhibitor, Pargyline, in order to augment the availability of (^3H)-dopamine for uptake into the dopaminergic processes and to enhance the difference between the amounts of (^3H)-dopamine accumulated in the dopaminergic and the indoleamine-accumulating neurons respectively.

3.2.1. Distribution of radioactive cell bodies and processes

The pattern of radioactivity observed both in the light and the electron microscope is similar to that seen in earlier studies with fluorescence histochemistry. The silver grains are concentrated in cell bodies mainly situated in the innermost cell row of the inner nuclear layer, but occasionally labelled cell bodies are also found in the ganglion cell layer and rarely in the inner plexiform layer. The cell bodies in the inner nuclear layer are among its largest ones with a horizontal diameter of 9.8 ± 0.38 μm (s.e.m) as compared to 8.9 ± 0.27 μm for the immediately surrounding cell bodies. The cell bodies of the ganglion cell layer appear to be as large as those of the inner nuclear layer, whereas those in the inner plexiform layer are smaller.

The radioactive label associated with the (^3H)-dopamine-accumulating processes in the inner plexiform layer is concentrated to three bands, roughly corresponding to sublaminae 1, 3 and 5, if the inner plexiform layer is divided into five equal sublaminae. The outermost band in the strongest, the middle one is weaker and the innermost one is weakest. Occasionally, the label is seen to be localized to a stout process emerging from a labelled cell body in the inner nuclear layer and descending into the inner plexiform layer.

The radioactively labelled neurons thus have the distribution of cell bodies and processes that is characteristic of amacrine cells and, more specifically, which is identical to that of the

dopaminergic neurons seen by fluorescence histochemical analysis of the endogenous dopamine content. This shows that the autoradiographic procedure has not labelled the indoleamine-accumulating neurons to any significant extent because they have a markedly different distribution of their processes. The ultrastructure of the labelled neurons is also typical of amacrine cells (Dowling and Boycott, 1966; Raviola and Raviola, 1967): an indented nucleus, processes consisting of varicosities and thin intervaricose segments, varicosities with unevenly distributed small synaptic vesicles, neurotubules, a few mitochondria, and synapses of the conventional type.

There are also large dense-cored vesicles scattered in both the cytoplasm of the cell bodies and in the processes of the dopaminergic neurons. They are of 90-160 nm diameter with a central, very dense core, and a clear space of about 5-12 nm between the core and the outer membrane. The silver grains tend to cluster around them, and in series of sections it is clearly seen that the presence of one or several large dense-cored vesicles in association with an aggregation of silver grains in a process is an unequivocal indicator of a labelled process, even in single sections.

3.2.2. Synaptic contacts

The (^3H)-dopamine-accumulating neurons are presynaptic to both the cell bodies of unlabelled amacrine cells and their processes. There are output synapses both onto varicosities and intervaricose segments of processes with amacrine cell characteristic and onto very thin intervaricose segments.

The (^3H)-dopamine-accumulating neurons are postsynaptic to unlabelled amacrine cell processes at conventional synapses. The number of such input synapses appears to be much smaller than the number of output synapses. In the previous study of the dopaminergic neurons in the retina of the rabbit, using a labelling method with 5,6-dihydroxytryptamine, a similar difference in num-

bers has also been found (Dowling and Ehinger, 1978 a). All of the input synapses from unlabelled processes in that study have been observed onto very thin intervaricose segments of the labelled dopaminergic processes, which contain few cell organelles, and therefore have few structures to show any signs of labelling. Thus, input synapses to such thin labelled processes may escape detection with falsely low numbers as a possible consequence. In this study, both intervaricose segments and small varicosities (0.4-0.6 μm) have been found with input synapses onto them. Synapses between (^3H)-dopamine-accumulating processes have, on the other hand, not been observed in the present study. A small number of such synapses were reported in the Dowling and Ehinger study (1978 a). The differences may represent random variations due to the small sample sizes. Serial synapses have been seen fairly frequently in both studies, involving both other amacrine cell processes and bipolar terminals. However, no direct contacts between dopaminergic and bipolar or ganglion cells have been observed with either type of labelling.

3.3. Comparison of dopaminergic neurons in different species

When the two labelling methods used for the investigation of the dopaminergic neurons in the Cynomolgus monkey and the rabbit are compared, it is clear that with some few restrictions they are interchangeable. The labelling with 5,6-dihydroxytryptamine has the advantage of high resolution, but the disadvantage of disrupting the normal ultrastructure of the labelled neurons and of requiring the prior removal of the indoleamine-accumulating processes in those species in which they are present. The autoradiographic method in combination with a monoamine oxidase inhibitor has the advantage of not requiring the use of the neurotoxic indoleamine to destroy the indoleamine-accumulating processes, thus leaving the normal cytoarchitecture undamaged. However, this method has inferior resolution due to the spread of silver grains, which can also obscure important structural details.

The dopaminergic neurons in the retinae of *Cynomolgus* monkeys and rabbits have many similarities. In both species, they are amacrine cells with the typical distribution of cell bodies and processes and with characteristic ultrastructural features. They contain the large dense-cored vesicles which have been associated with aminergic neurons (Hökfelt, 1968; Tranzer and Richards, 1971; Palay and Chan-Palay, 1975). Most of their dendrites ramify close to the junction of the inner nuclear and inner plexiform layers; hence their earlier name "adrenergic junctional cells" (Ehinger, 1966 b). They have output synapses onto non-dopaminergic amacrine cell bodies in the inner nuclear layer and onto processes of amacrine cells as they enter the inner plexiform layer. The dopaminergic neurons are pre- and postsynaptic only to other amacrine cells, forming interamacrine connections. The same synaptic organization has been reported for the dopaminergic amacrine cells of the cat (Pourcho, 1981 a). Furthermore, the dopaminergic interplexiform cells, present in the retinae of teleost fish and New World monkeys, have been shown to have similar interamacrine connections in the inner plexiform layer (Dowling and Ehinger, 1975, 1978b; Dowling, Ehinger and Florén, 1980).

3.4. Function of dopaminergic neurons

Little is known concerning the function of the dopaminergic amacrine cells. Dopamine has a general inhibitory effect of the firing of retinal ganglion cells in the cat (Straschill and Perwein, 1975). The dopaminergic interplexiform cells in cyprinid fish may regulate the center-surround antagonism in the outer plexiform layer (Hedden and Dowling, 1978), but no corresponding experiments have yet been possible in the inner plexiform layer of mammals. In both the rabbit, cat and *Cynomolgus* monkey, the dopaminergic processes ramify mainly in the outermost parts of the inner plexiform layer, in which OFF signals in cats and rabbits have been shown to be mediated from hyperpolarizing bipolar cells to OFF ganglion cells (Famiglietti and Kolb, 1976; Nelson, Famiglietti and Kolb, 1978; Bloomfield and Miller, 1981). Possibly,

then, dopaminergic neurons are engaged in regulating the OFF responses. It seems somewhat less likely that they participate in the direct generation of the OFF responses because they do not connect directly to bipolar or ganglion cells.

3.5. Indoleamine-accumulating neurons in rabbits (III), cats (IV) and cyprinid fish (V)

The indoleamine-accumulating neurons are identified by means of their high affinity uptake for indoleamines (Ehinger and Florén, 1978). They were first seen in retinae processed according to the Falck and Hillarp method after the accumulation of the exogenous indoleamine, 5,6-dihydroxytryptamine (Dowling and Ehinger, 1975; Ehinger and Florén, 1976). On the light microscope level, they have subsequently also been demonstrated in several species using autoradiography with (^3H)-5-hydroxytryptamine (Ehinger, Hansson and Tornqvist, 1981; Ehinger, Florén and Tornqvist, 1982; Marc, 1980). There are several peculiarities associated with these neurons. They were originally thought to use 5-hydroxytryptamine as their transmitter, but endogenous 5-hydroxytryptamine cannot be repeatedly demonstrated with the Falck and Hillarp method except during a short stage in the embryonic development of chicken (Hauschild and Laties, 1973). More extensive biochemical investigations have later shown that the neurotransmitter is unlikely to be 5-hydroxytryptamine in the retina of mammals (Florén, 1978; Ehinger, Hansson and Tornqvist, 1981). However, in several submammalian species such as chicken, lizard, frog and goldfish, there is chromatographical and immunocytochemical evidence that the transmitter is actually 5-hydroxytryptamine (Osborne, Nesselhut, Nicholas and Cuello, 1981; Osborne, Nesselhut, Nicholas, Patel, Cuello and Milstein, 1981; Ehinger, 1982).

In this study, the 5,6-dihydroxytryptamine labelling method has been used to label the indoleamine-accumulating neurons in the retinae of the rabbit, cat and cyprinid fish. The dopaminergic processes have been removed by treatment with 6-hydroxydopamine to avoid interference.

3.5.1. Indoleamine-accumulating neurons in rabbits

The indoleamine-accumulating neurons in the retina of the rabbit are seen both in the fluorescence microscope and the electron microscope to have most of their cell bodies in the innermost cell row of the inner nuclear layer and their processes in the inner plexiform layer. The density of the indoleamine-accumulating neurons is 8.8 ± 0.75 (s.e.) cells per mm² section of the retinae, which is about twenty times more numerous than the dopaminergic ones (Ehinger and Åberg, 1981). The indoleamine-accumulating neurons are also more numerous in the central retina than in the periphery. A small number of cell bodies are found further outwards in the inner nuclear layer, a few are seen in the ganglion cell layer and a very small portion of the cell bodies are situated in the inner plexiform layer. The indoleamine-accumulating processes ramify mainly in the innermost sublayer of the inner plexiform layer close to the junction between the inner plexiform and ganglion cell layers. Two weaker bands can be observed in the middle and outermost parts of the inner plexiform layers, the outermost one being the densest of these two.

The indoleamine-accumulating processes consist of varicosities and alternating intervaricose segments, and in the electron microscope they have amacrine cell characteristics including conventional output synapses. The ultrastructural changes produced by the labelling drug are increased density of the cell membrane, particularly in the synaptic and junctional regions and of the cell organelles. The small synaptic vesicles often contain an electron dense spot and the large dense-cored synaptic vesicles have central cores of increased density. The cell organelles are often somewhat distorted.

The indoleamine-accumulating varicosities in the innermost sublayer of the inner plexiform layer aggregate around big bag-like bipolar terminals, which because of their position and ap-

pearance are considered to be rod bipolar cells. The indoleamine-accumulating processes and the bipolar terminals are pre- and postsynaptic to each other, and reciprocal synapses are so common so as to be considered the rule. The output synapses to the bipolar terminals consist of several aggregations of small synaptic vesicles which cluster around dense globules of 80-120 nm diameter. These structures have been called globular clusters. The pre- and postsynaptic membrane densities and the dense material in the synaptic cleft are similar to those found at other conventional synapses. At the ribbon synapses of the rod bipolar terminals, the other postsynaptic element in the dyad with an indoleamine-accumulating process has always been an unlabelled amacrine cell process.

The indoleamine-accumulating neurons are also postsynaptic to amacrine cell processes mainly in the outermost sublamina of the inner plexiform layer, where input synapses have been seen onto the labelled processes a short distance from their origin at a labelled cell body. Output synapses from indoleamine-accumulating processes onto unlabelled amacrine cell processes appear to be rare. Most of the ones detected have been in the innermost part of the inner plexiform layer.

The contacts between two indoleamine-accumulating processes are often desmosome-like, but a few junctions resembling synapses have also been observed.

3.5.2. Indoleamine-accumulating neurons in cats

The indoleamine-accumulating neurons in the retina of the cat have also been studied in the fluorescence and electron microscope after the uptake of 5,6-dihydroxytryptamine. In addition, they can be observed in the light microscope using phase contrast optics in tissue prepared for electron microscopy. The indoleamine-accumulating neurons appear black and dense with this technique and especially the pattern of ramification of the indoleamine-accumulating processes in the inner plexiform layer is clearly seen.

The cell bodies of indoleamine-accumulating neurons are situated in the innermost two cell rows of the inner nuclear layer (83%), in the ganglion cell layer (17%) and in the inner plexiform layer (about 0.5%). The average density of cell bodies is 20.9 ± 0.99 (s.e.m) per mm² section of the retina, but there is a difference in the density of cell bodies in the center and in the periphery in that they are roughly three times more numerous centrally.

The indoleamine-accumulating processes form three to five thin bands in the outer half of the inner plexiform layer, whereas they ramify rather diffusely in the inner half. The ultrastructural changes in the labelled cells are similar to those described in the retina of the rabbit. The synapses of the indoleamine-accumulating neurons in the cat are of the conventional type with all the characteristics of amacrine cells. Globular clusters have not been found.

The synaptic partners of the indoleamine-accumulating neurons in the inner plexiform layer of the retina of the cat have been identified on the basis of the criteria established by earlier investigators (Boycott and Kolb, 1973; Kolb and Famiglietti, 1974 and 1976; Kolb, 1979). The indoleamine-accumulating neurons predominantly have synaptic contacts with rod bipolar terminals deep in sublamina b where the labelled processes cluster around them. These synapses are probably always reciprocal, and the postsynaptic dyads consist of two amacrine cell processes, of which one is indoleamine-accumulating. More than half of the processes with reciprocal synapses with rod bipolar terminals deep in sublamina b appear to be indoleamine-accumulating. The output synapses to the rod bipolar terminals are fairly inconspicuous in the synaptic structures.

Further out in sublamina b, there are also output synapses to bipolar terminals, most of which belong to rod bipolar cells. A few synapses have been observed in the peripheral retina with

bipolar terminals also engaging in synaptic contacts with ganglion cell dendrites. These few bipolar terminals may be from invaginating cone bipolar cells. In sublamina a, the indoleamine-accumulating neurons are pre- and postsynaptic to flat cone bipolar terminals.

There are also input and output synapses with other amacrine cell processes throughout the inner plexiform layer. A few synapses between labelled processes have been detected in sublamina a and the outermost part of sublamina b. There are a small number of output synapses to ganglion cell dendrites. Serial synapses and convergent synapses are relatively frequent.

3.5.3. Comparison of indoleamine-accumulating neurons in rabbits and cats

The indoleamine-accumulating amacrine cells of the rabbit and the cat have several features in common. Most of their processes cluster around rod bipolar terminals in the innermost parts of the inner plexiform layer. Their main synaptic arrangement is reciprocal synapses with these rod bipolar cells, forming local feed-back loops. There is, however, a difference in that the output synapses to the rod bipolar terminals in the rabbit are characterized by prominent synaptic structures, the globular clusters. The corresponding synapses in the cat have indistinct synaptic structures of "punctate" appearance. In both animals, the indoleamine-accumulating processes are both pre- and postsynaptic to other amacrine cells, but in the cat there are additional contacts with cone bipolar cells and ganglion cells. Because of the distribution of indoleamine-accumulating processes and their synapses, it is assumed that they mainly play a role in the rod pathway, but that they probably integrate signals of a wide variety both in terms of the ON and OFF pathways and of connections between different classes of retinal neurons.

3.5.4. Indoleamine-accumulating neurons in goldfish and carp

Already the distribution of the indoleamine-accumulating processes in the retinae of goldfish and carp indicates differences between these population of neurons in the mammalian and cyprinid retina. Both in the fluorescence and the electron microscope it is clearly seen that the indoleamine-accumulating cell bodies are fairly small in size, and even though they are usually situated in the innermost half of the inner nuclear layer, they are often found further outwards from the innermost cell row. Their processes ramify in three bands in the inner plexiform layer, and the outermost one is much denser than the other two.

The labelled neurons have the usual characteristics of amacrine cells, both in terms of ultrastructure and synapses. The ultrastructural changes produced by the labelling drug are also similar to those of the mammalian retinae and described above.

The indoleamine-accumulating processes are mainly pre- and postsynaptic to other amacrine cells. Input synapses from unlabelled amacrine cells are frequent, whereas output synapses are somewhat less commonly seen. There are a few output synapses to cell bodies in the innermost cell row of the inner nuclear layer. Synaptic contacts, both output and input, with bipolar terminals are present throughout the inner plexiform layer, though in a rather small number. The bipolar terminals are of varying size and presumably of several types (Ishida, Stell and Lightfoot, 1980). Contacts with suspected ganglion cell dendrites have been seen, but they are scarce.

With moderate to high doses of 5,6-dihydroxytryptamine, a set of rather large labelled processes is seen in the middle sublamina of the inner plexiform layer. In addition to conventional synapses, these processes have a special type of conventional synapse with two postsynaptic elements, usually two amacrine cell processes. This kind of contact has been called a branched conventional synapse.

The indoleamine-accumulating neurons ramify mainly in the outer half of the inner plexiform layer, where OFF signals are mediated from hyperpolarizing bipolar cells to OFF ganglion cells (Famiglietti, Kaneko and Tachibana, 1977). Since their connections with amacrine cells dominate and they contact both bipolar and amacrine cells throughout the inner plexiform layer, they are likely to act both on the ON and OFF pathways.

3.6. Comparison of dopaminergic and indoleamine-accumulating neurons

From the studies on the dopaminergic and indoleamine-accumulating retinal neurons, it is clear that pharmacologically different subpopulations may have different synaptic organization in a single animal species. In the rabbit, for example, the dopaminergic processes form interamacrine connections only, whereas the indoleamine-accumulating neurons mainly have reciprocal synapses with rod bipolar terminals. The dopaminergic amacrine cells are the only ones known to contact other amacrine cells exclusively. The indoleamine-accumulating, the GABAergic and the glycinergic amacrine cells contact both bipolar and amacrine cells (Pourcho, 1980, 1981 b; Vaughn, Famiglietti, Barber, Saito, Roberts and Ribak, 1981). In the goldfish and carp, the dopaminergic neurons are interplexiform cells, which are pre- and postsynaptic to other amacrine cells in the inner plexiform layer and presynaptic to bipolar and horizontal cells in the outer plexiform layer (Dowling and Ehinger, 1975, 1978 b). The indoleamine-accumulating neurons in the same fish are amacrine cells with predominantly interamacrine connections. A few contacts with bipolar cell and suspected ganglion cells have also been seen.

In addition, one pharmacologically distinct subpopulation may vary in its synaptic organization from species to species. While the dopaminergic neurons in the retinae of Cynomolgus monkeys and rabbits are amacrine cells with interamacrine connections, they are in teleost fish and New World monkeys interplexiform cells, which are pre- and postsynaptic to amacrine cells in the inner

plexiform layer, and presynaptic to bipolar and horizontal cells in the outer plexiform layer (Dowling and Ehinger, 1975, 1978 b; Dowling, Ehinger and Florén, 1980). As already mentioned, the indoleamine-accumulating neurons mainly have reciprocal synapses with rod bipolar terminals in the innermost part of the inner plexiform layer in the rabbit, cat and Cebus monkey (Dowling, Ehinger and Florén, 1980), whereas they mainly have synaptic contacts with amacrine cells in the outermost part of the inner plexiform layer in the goldfish and carp.

The function of the indoleamine-accumulating neurons is not very well known. In all the animals investigated they ramify in both the ON and OFF layers of the inner plexiform layer, even though they have the majority of their processes and synapses in the OFF layer in the mammals and in the ON layer in cyprinid fish. The effects of 5-hydroxytryptamine on the responses of ganglion cells have been too complex to allow any conclusions about the possible function of the indoleamine-accumulating neurons (Ames and Pollen, 1969; Straschill and Perwein, 1975).

3.7. Synapses of the inner plexiform layer in cyprinid fish

Two main types of synapses are recognized in the inner plexiform layer of the vertebrate retina: the ribbon synapse and the conventional synapse.

3.7.1. The ribbon synapse

The ribbon synapse has a presynaptic pentalaminar ribbon surrounded by a shell of small synaptic vesicles and prominent dense deposits on the presynaptic membrane. There are usually two postsynaptic elements in a dyad arrangement. The postsynaptic membrane deposits are also very prominent, and there is dense material in the synaptic cleft. The ribbon synapses are characteristic of bipolar cells (Dowling and Boycott, 1966; Goodland, 1966; Raviola and Raviola, 1967; Witkovsky and Dowling, 1969). In several species, variations in the synaptic arrangement of ribbon

synapses have been reported with not only two but, more rarely, one, three or four postsynaptic elements (Allen, 1969; Dubin, 1970; Witkovsky and Stell, 1973; Wong-Riley, 1974; Dowling, personal communication). These ribbon synapses have been called monadic, triadic and tetradic, respectively. Bipolar terminals with both ribbon synapses and conventional synapses have been described by two investigators (Allen, 1969; Wong-Riley, 1974).

3.7.2. The conventional synapse

The conventional synapse is characterized by a presynaptic aggregation of small synaptic vesicles, some dense deposits on the presynaptic membrane, dense material in the synaptic cleft and a varying amount of dense material on the postsynaptic membrane. Only conventional synapses with one postsynaptic element have previously been described.

Variations in the synaptic structures of the conventional synapse have been reported. In the retina of the cat, the AII amacrine cell (now called A7) has "punctate" output synapses from its lobular appendages to the processes of ganglion, flat cone bipolar and amacrine cells (Kolb, 1979; Kolb, Nelson and Mariani, 1981). These synapses only have a very inconspicuous cluster of small synaptic vesicles close to dense deposits on the presynaptic membrane, and the pre- and postsynaptic membrane densities are weak. Furthermore, the output synapses from the indoleamine-accumulating processes to the rod bipolar terminals in the innermost part of the inner plexiform layer in the retina of the rabbit are characterized by several clusters of small synaptic vesicles which aggregate around dense globular structures of about 80-120 nm diameter. These structures have been called globular clusters as mentioned above.

3.7.3. Conventional synapses in cyprinid fish

The conventional synapses with one postsynaptic element belong to either of two subtypes in the normal retinae of goldfish and carp. Certain monadic conventional synapses have one single

aggregation of small synaptic vesicles on the presynaptic side. However, there are also monadic conventional synapses which resemble the synapses with globular clusters in the retina of the rabbit. These synapses have several presynaptic clusters of small synaptic vesicles, which surround dense globules of about 100 nm diameter. The pre- and postsynaptic membrane densities and the material in the synaptic cleft have the same appearance as in the other type of conventional synapse.

3.7.4. The branched conventional synapse

During the investigation of the indoleamine-accumulating neurons in the retina of goldfish and carp, a subset of indoleamine-accumulating processes were seen, which had both conventional output synapses of the usual type described above and conventional output synapses with two postsynaptic elements. These synapses have subsequently been detected in the normal retina of both goldfish and carp and they have been called branched conventional synapses.

The branched conventional synapses are characterized by a protrusion on the part of the presynaptic process. At the tip of the protrusion there is a dense globule of a diameter of about 100 nm. The globule appears granular in structure, and radial fibrils extend from it into the cytoplasm. An inconspicuous cluster of small synaptic vesicles surround the central globule, and there is material around it on the presynaptic membrane. The synaptic cleft is clearly widened to about 20 nm. There is also dense material in the cleft and on the postsynaptic membranes of the two postsynaptic elements. The widening of the cleft is concurrent with the dense material in it and with the pre- and postsynaptic membrane densities. The structures of the branched conventional synapses can be followed only through three to five consecutive sections of about 50-60 nm thickness.

The branched conventional synapses are present in all sublaminae of the inner plexiform layer, but they appear to be most common in the middle part of that layer. The most frequent combinations of postsynaptic elements are two amacrine cell processes, one amacrine cell varicosity together with one thin intervaricose segment, and two thin intervaricose segments. However, both bipolar processes and suspected ganglion cell dendrites have been seen to be postsynaptic at branched conventional synapses.

Processes with up to three branched conventional synapses have been detected. These processes have amacrine cell characteristics, and several of them also make conventional monadic output synapses like amacrine cells. The processes with branched conventional synapses are therefore thought to be amacrine cell processes.

3.7.5. Function of branched conventional synapses

The arrangement of the branched conventional synapses appears to indicate a spreading of information. Furthermore, the frequent involvement of thin intervaricose segments, which have different electrophysiological properties than the intervaricose segments, may have a special functional significance. It is conceivable that the propagation of a signal along a neural process can be efficiently controlled by synapses acting on the intervaricose segments.

4. GENERAL SUMMARY

The synaptic connections of the dopaminergic retinal neurons of Cynomolgus monkeys and rabbits were studied in the electron microscope. In the Cynomolgus monkeys, a labelling method with 5,6-dihydroxytryptamine was used, whereas an autoradiographic technique with a monoamine oxidase inhibitor and (^3H)-dopamine was used in the rabbits.

The labelling method with 5,6-dihydroxytryptamine altered the normal ultrastructure of the dopaminergic neurons, but their synaptic structures were still easily recognized. The autoradiographic method left the ultrastructure of the labelled neurons unchanged, and large dense-cored vesicles could therefore be seen to be associated with the dopaminergic neurons in the rabbits. Other morphological characteristics could also be seen.

The distribution of dopaminergic perikarya in the inner nuclear layer and dopaminergic processes in the inner plexiform layer was consistent with that of amacrine cells in both Cynomolgus monkeys and rabbits. Furthermore, the dopaminergic processes had output synapses of the conventional kind characteristic of amacrine cells. In both animals, the dopaminergic processes were presynaptic to amacrine cell bodies and both pre- and postsynaptic to other amacrine cell processes. The number of output synapses was larger than the number of input synapses. Serial and convergent synaptic arrangements were common.

The synaptic organization of the indoleamine-accumulating neurons in the retinae of rabbits, cats, goldfish and carp were also investigated in the electron microscope. The neurons were labelled with 5,6-dihydroxytryptamine after the otherwise interfering dopaminergic processes had been destroyed.

The indoleamine-accumulating neurons of the three species had a distribution of perikarya and processes like that of amacrine cells. They also had synapses of the conventional kind. In the rabbit and the cat, the indoleamine-accumulating processes had

mainly reciprocal synapses with rod bipolar terminals in the innermost part of the inner plexiform layer. They were, however, also pre- and postsynaptic to other amacrine cells, and in the cat, they had synaptic contacts with cone bipolar cells and the dendrites of ganglion cells.

The indoleamine-accumulating neurons of the goldfish and carp were mainly pre- and postsynaptic to other amacrine cells, even if contacts with bipolar terminals were also seen.

In the retinae of goldfish and carp, a subset of indoleamine-accumulating processes were discovered in the middle part of the inner plexiform layer that had a previously not recognized type of conventional output synapse with two postsynaptic elements. This synapse was called a branched conventional synapse. It was found and investigated in the retinae of normal goldfish and carp. Its presynaptic structures were found to be similar to those seen in certain conventional synapses with presynaptic "globular clusters" and only one postsynaptic element.

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SYNAPTIC ORGANIZATION OF THE DOPAMINERGIC NEURONS IN THE RETINA OF
THE CYNOMOLGUS MONKEY

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ABSTRACT

Dopaminergic neurons have previously been demonstrated in the retina of Cynomolgus monkeys (Macaca fascicularis) by fluorescence microscopy. These neurons take up 5,6-dihydroxytryptamine, which alters their ultrastructure, and this technique has been used in the present study to identify the dopaminergic retinal neurons in the electron microscope. There are no indoleamine-accumulating neurons in the Cynomolgus monkey retina to interfere with the analysis of the dopaminergic cells. The dopaminergic neurons have their cell bodies among those of the amacrine neurons in the inner nuclear layer. Their processes ramify mainly in the outermost sublayer of the inner plexiform layer. However, dopaminergic processes can be found occasionally to extend into the middle part of the inner nuclear layer and rarely into the innermost parts of the inner plexiform layer. All their output synapses are of the conventional kind. They appear to form synaptic connections with other amacrine neurons only, indicating that the dopaminergic amacrine cells in the retina of the Cynomolgus monkey are inter-amacrine neurons.

KEY WORDS

Retina - Synapses - Dopamine - Electron microscopy - Cynomolgus monkey

INTRODUCTION

The distribution of the dopamine-containing cells and their processes in the retina can be readily visualized with the histochemical method of Falck and Hillarp^{1,2}. In all species investigated so far they have their perikarya in the inner nuclear layer along the junction of the inner nuclear and inner plexiform layers. Occasionally, a dopamine-containing perikaryon can be found in the ganglion cell layer or rarely in the inner plexiform layer.

In most species the processes of the dopaminergic neurons are found mainly within the inner plexiform layer and they usually ramify near the junction of the inner nuclear and inner plexiform layers. This is the case in the retinas of the mouse, rat, cat, of some monkeys such as the *Cynomolgus* (an Old World monkey) and of man³. In other animals the dopaminergic processes form two or three sublayers in the inner plexiform layer. This pattern of ramification is found in the toad, mudpuppy, pigeon, chicken, guinea pig, rabbit, and baboon, among others³. In many species, including most mammals, the dopamine-containing processes also extend into the inner nuclear layer³. Their numbers and distribution in this layer vary from species to species. Most commonly, there are only a few processes between the non-dopaminergic perikarya in the innermost half of the inner nuclear layer, but in several New World monkeys they form a plexus of fibers within the inner nuclear layer^{4,5,6}.

In teleost fish and several New World monkeys such as the *Cebus*, the dopaminergic neurons have processes that extend widely both in the inner and the outer plexiform layers. These neurons make abundant synapses in the outer plexiform layer, on horizontal and bipolar cells, and for this reason they are classified as a separate kind of retinal neuron, the interplexiform cell^{4,5,7,8,9,10}. Interplexiform cells have also been demonstrated in

other animal species such as the dolphin, rat, cat, rabbit and Rhesus monkey^{6,11,12,13,14,15}. However, the interplexiform cells of these animals are not visualized with the technique of Falck and Hillarp and consequently they are not believed to be dopamine-containing.

The dopaminergic cells and their processes are not ordinarily identifiable in the electron microscope. Therefore, to analyze the synaptic connections of these cells, the neuronal processes must be labeled. Dopaminergic cells will actively accumulate both dopamine and certain analogous amines such as alpha-methylnoradrenaline, 6-hydroxydopamine or 5,6-dihydroxytryptamine^{9,16,17} and certain of these analogues will alter the fine structure of the neurons. Of these analogues 5,6-dihydroxytryptamine has the advantage of producing distinctive ultrastructural changes in the dopaminergic processes as well as increasing the intensity of their fluorescence^{8,9,10,16,17}.

However, 5,6-dihydroxytryptamine is taken up not only by the dopaminergic retinal neurons, but also by the indoleamine-accumulating neurons present in the retinas of many animal species^{10,18,19} and this complicates the analysis of the dopaminergic cell synaptic organization. On the other hand, there are no indoleamine-accumulating retinal neurons in the Cynomolgus monkey¹⁷. Hence, to study the distribution of the dopaminergic neurons in the fluorescence microscope, or their ultrastructure in the electron microscope, 5,6-dihydroxytryptamine may be used.

The Cynomolgus monkey is of interest not only because it is a primate but also because its retina is particularly similar to that of man^{17,20}. It is obvious that care must be taken not to draw too generalized conclusions about man from facts obtained from Cynomolgus monkeys. Nevertheless, the similarities give a special significance to the elucidation of the synaptic organization of the dopaminergic retinal neurons of the Cynomolgus monkey.

MATERIALS AND METHODS

Three adult *Cynomolgus* monkeys (*Macaca fascicularis*) were used. All injections were given intravitreally with a precision syringe. Four eyes were injected with 50 ug 5,6-dihydroxytryptamine. The drug was dissolved in 50 ul 0.9% NaCl with ascorbic acid added as antioxidant (1 mg/ml). One control eye did not receive any injection, while another was injected with the solvent only. The eyes were enucleated 4 hours after the intravitreal injection. The posterior segments of experimental and control eyes were prepared for electron microscopy by fixation in 2% OsO₄ in veronal acetate buffer (pH 7.6-7.8) containing 28 mM barbital sodium, 28 mM sodium acetate, 66 mM sucrose and 1.8 mM CaCl₂ for 1 hour in an ice bath and for another half hour at room temperature²¹. The tissue was then dehydrated and embedded in Epon 812, sectioned and stained with uranyl acetate and lead citrate according to conventional methods. Tissue blocks from at least three different parts of the posterior segments were taken. The sections were analyzed in a Philips 300 and JEOL 100 B electron microscope.

As additional controls, pieces of retina from all eyes were processed for fluorescence microscopy according to the method of Falck and Hillarp².

5,6-dihydroxytryptamine creatinine sulphate was obtained from the Regis Chemical Co., Morton Grove, Illinois, U.S.A.

RESULTS

Light microscopy

The fluorescent dopaminergic cell bodies were situated almost entirely in the innermost row of amacrine cell bodies in the inner nuclear layer (Fig. 1a and b). There were fluorescent cell bodies occasionally seen in the ganglion cell layer. These "alloganglion" cells²² constituted no more than five to ten percent of the number of dopaminergic cell bodies found in the innermost part of the inner nuclear layer. Dopaminergic cell bodies were also very rarely detected in the inner plexiform layer ("eremite" cells²²).

Nearly all of the dopaminergic neuronal processes were confined to the outermost part of the inner plexiform layer. However, dopaminergic processes were sometimes observed extending between non-fluorescent cell bodies in the innermost part of the inner nuclear layer and occasionally surrounding such a cell body. Only very rarely could a dopaminergic process be seen further out in the inner nuclear layer or in the inner parts of the inner plexiform layer.

Electron microscopy

Ultrastructural alterations:

The ultrastructural changes produced in the Cynomolgus monkey by 5,6-dihydroxytryptamine were similar to those described in previous studies using this labeling method^{8,9,10,17,23}. For example, the membranes of the small synaptic vesicles (20-40 nm) in the labeled processes usually showed increased density and within the vesicles an eccentrically located dense spot of material was often present. The vesicles were also sometimes elongated or flattened in shape (Fig. 2a and b). Large vesicles (50-70 nm) with an enhanced electron dense core were frequently present in the processes. Sometimes these large vesicles were completely filled with electron-dense material, but at other times their outer

membrane was distorted into a long process or was "ballooned" to one side (Fig. 2 and 3a).

The cell membrane of labeled neurons usually did not show any increased electron density except in synaptic or other junctional regions (Fig. 4 and 5). The increase in electron density of mitochondrial membranes was, however, frequently quite marked and the mitochondria were often swollen (Fig. 4, 5 and 6). Most labeled processes seemed to have their cell structure well preserved in spite of the above mentioned changes in the organelles (Fig. 7 and 8). Other processes had obvious signs of degeneration. Some of them were swollen, and in those the usual organelles including synaptic vesicles were scarce. In others tight clusters of small synaptic vesicles were found in one part of the process. The rest of the varicosity was then empty (Fig. 6). Other processes seemed to be shrunken. Shrunken varicosities were usually filled with small synaptic vesicles of increased electron density (Fig. 9a).

In addition to a variation in the labeling from process to process, there was also variability in the labeling within each given neuronal process. A dopaminergic process which in one section contained both mitochondria and synaptic vesicles showing alterations could in an adjacent section display only unlabeled organelles (Fig. 3a and b). Also, in a process in one particular section, the mitochondria could be labeled and the synaptic vesicles unlabeled, whereas in an adjacent serial section the mitochondria were unlabeled and the synaptic vesicles labeled.

Distribution of labeled processes

The labeled processes consisted of the usual varicosities alternating with thin intervaricose segments. When cut in cross-section the labeled intervaricose segments were difficult to detect. The varicosities were generally of intermediate size, most with a diameter of 0.3 - 0.7 μm .

The distribution of the labeled dopaminergic processes was in agreement with the fluorescence microscopical findings. The vast majority of the dopaminergic processes were found rather evenly distributed in the outermost sublayer of the inner plexiform layer, near the junction between the inner plexiform and inner nuclear layers. At times labeled varicosities were seen in the vicinity of retinal capillaries, but they were never to be found in direct contact with capillary structures. On occasion degenerating labeled processes were observed inside glial cells in the outer parts of the inner plexiform layer.

Processes were occasionally found between and on the outside of cell bodies in the innermost row of amacrine cell bodies in the inner nuclear layer (Fig. 10), but they were not seen further outwards. Labeled processes were extremely rare in the middle and inner parts of the inner plexiform layer. No labeled processes were detected in the outer plexiform layer.

Synaptic structures

All synapses made by the dopaminergic processes were of the conventional kind with a cluster of small synaptic vesicles on the presynaptic side of the junction. These vesicles were occasionally labeled, but sometimes not. The large synaptic vesicles with a dense core were at times seen in close proximity to a synapse, but more often they appeared to be distributed in the varicosity without any special relationship to the synaptic structures. On the presynaptic membrane there was always an aggregation of electron dense material with either a fibrillar, granular or flocculent appearance. The presynaptic membrane itself showed some slight increased electron density. The synaptic cleft sometimes appeared slightly widened and it usually contained electron dense material (Fig. 2, 4, and 6). On the postsynaptic membrane there was also often an aggregation of electron dense material, usually less than on the presynaptic side but of the same general appearance as the presynaptic membrane material.

The output synapses were found in the varicosities of the dopaminergic processes and contacts were made onto the varicosities of unlabeled neurons as well as onto their intervaricose segments. In at least five instances, output synapses from labeled varicosities were seen to contact an amacrine process adjacent to its cell body (Fig. 8a and b). Several of the dopaminergic output synapses were onto varicosities, which themselves contained a conventional synapse (Fig. 6 and 7). In these cases the postsynaptic neurons were likely to be amacrine cells²⁴. Synapses from labeled dopaminergic processes onto unlabeled amacrine perikarya in the innermost row of cell bodies in the inner nuclear layer were also frequently seen (Fig. 7).

At many synapses, the postsynaptic neuron was hard to identify with certainty, but most of them had characteristics considered typical of amacrine neurons, for example, unevenly scattered synaptic vesicles and few other cytoplasmic organelles such as mitochondria and neurotubules in the processes²⁴. In no case did the postsynaptic neuron have features similar to those of bipolar terminals such as bag-like shape, numerous evenly distributed synaptic vesicles and synaptic ribbons²⁴. Also, none of the postsynaptic processes had the appearance of ganglion cell dendrites with abundant ribosomes, vesicular endoplasmic reticulum and absence of synaptic vesicles²⁴.

A smaller number of contacts between labeled processes were observed. A few of those contacts resembled the typical amacrine synapses described above. In other cases the contacts between two labeled processes more closely resembled desmosomes with very symmetrical aggregations of fibrillar electron dense material and increase in membrane electron density (Fig. 5a and b). The intercellular cleft also contained electron dense material. Vesicular structures were occasionally seen in the processes close to a

junction like this, but they were not arranged in presynaptic clusters. These junctions were also seen occasionally between labeled and unlabeled processes (Fig. 3a and b, 9a and b), but they tended to be more inconspicuous and asymmetric in that the deposition of material on the cell membrane of the labeled process was normally larger. Electron dense material was observed in the intercellular space at these junctions. A majority of the unlabeled elements were clearly neurons with amacrine characteristics, but one or two of them were presumed to be glial processes.

A smaller number of synapses onto labeled processes were detected. Of the synapses observed involving labeled processes only about 5% were input synapses. Furthermore, these synapses were inconspicuous and difficult to find. The presynaptic cluster of synaptic vesicles was always of the conventional kind, but usually only a few small synaptic vesicles were observed close to the presynaptic membrane. In most input synapses there was a deposition of electron dense material on both the pre- and postsynaptic membranes, but the presynaptic deposition was always larger. Both membranes occasionally had slightly increased electron density and the synaptic cleft contained fibrillar material (Fig. 3a and b). Because of their synaptic structure, all presynaptic contacts were identified as likely to be coming from amacrine cells. The presynaptic process was usually a small-sized varicosity whereas the postsynaptic dopaminergic processes were mostly middle-sized varicosities.

Synaptic organization

Serial synapses involving synapses from a dopaminergic process onto an amacrine cell process or perikaryon and from that element onto another unlabeled process were rather frequent (Fig. 6). There were also serial synapses involving a bipolar terminal as the third process (Fig. 7).

Single dopaminergic processes were seen in serial sections to make two separate synaptic contacts onto the same amacrine process close to its cell body, or onto the same unidentified unlabeled process. In other instances, two labeled processes with synapses onto the same amacrine process were seen in one section. Conversely, labeled and unlabeled amacrine processes were observed to have synapses onto the same unlabeled varicosity (Fig. 4). Finally, there were dopaminergic processes with synaptic output onto two separate unlabeled varicosities as well as onto one unlabeled process and an amacrine cell body. Only one or two suspected reciprocal synapses between labeled and unlabeled amacrine cells were observed.

DISCUSSION

The technique of labeling aminergic retinal neurons with 5,6-dihydroxytryptamine produced in this study enhanced fluorescence and ultrastructural changes similar to those described in earlier studies^{8,9,10,17,23}. Compared with autoradiographic techniques, the labeling with 5,6-dihydroxytryptamine has the advantage of superior resolution, but one of the greatest advantages is the opportunity to study both the distribution of labeled neurons and their processes with fluorescence microscopy and the ultrastructure and synaptic connections of the same neurons in one and the same retina. The distribution of labeled perikarya and processes found in this study is in agreement with the findings of earlier fluorescence microscopy studies of non-treated *Cynomolgus* monkey retinas as well as 5,6-dihydroxytryptamine-treated retinas^{17,20,22,25}.

Since the distribution of labeled neurons and their processes were the same with both fluorescence and electron microscopy, it is clear that the labeled neurons were dopaminergic and that no other neurons have been labeled with this experimental procedure. In addition, light microscope autoradiography after incubation of

Cynomolgus monkey retinas in (^3H)-5-hydroxytryptamine has shown radioactivity only in a single layer in the inner plexiform layer near the junction between the inner nuclear and plexiform layers, and radioactive cell bodies only among the amacrine cells in the innermost cell row of the inner nuclear layer²⁶. Thus, autoradiography confirms the earlier findings that there are no indoleamine-accumulating neurons in the retina of this monkey.

Nearly all the dopaminergic neurons in the Cynomolgus monkey retina belong to a cell type which was originally called the "adrenergic junctional" cell⁵. All available evidence indicates that the neurotransmitter of these neurons is dopamine. Their perikarya are situated among those of the amacrine cells in the inner nuclear layer, and apart from some ramifications in the inner nuclear layer, their processes are confined to the inner plexiform layer. Also like amacrine cells, the dopaminergic neurons make synapses of the conventional kind. Thus, the dopaminergic neurons in the Cynomolgus monkey have all the characteristics of amacrine cells, and it seems preferable to call them dopaminergic amacrine cells. The processes of the infrequent dopaminergic "alloganglion" and "hermit" cells²² observed in most species including the Cynomolgus monkey²⁰ also appear to ramify in the inner plexiform layer with the processes of the dopaminergic amacrine cells^{3,10,20,25}. In the Cynomolgus monkey, a process can often be seen to extend from a dopaminergic "alloganglion" cell body through the inner plexiform layer to join the layer of dopaminergic processes in the outermost part of the inner plexiform layer (see 20, fig. 8, page 48). The distribution of dopaminergic processes in the vicinity of dopaminergic "alloganglion" and "hermit" cells is similar to the distribution found in regions where these cells are not present. Furthermore, since only synapses between labeled processes and other amacrine elements have been observed in this study, it appears that the dopaminergic "alloganglion" and "hermit" cells have a synaptic organization similar

to that of the dopaminergic amacrine cells. Therefore, at least some of the dopaminergic "alloganglion" and "eremite" cells may be regarded as displaced amacrine cells.

In teleost fish and several New World monkeys the interplexiform cells are dopaminergic^{4,5,7,8,9,10}. The interplexiform cells have been extensively studied in the goldfish and in the Cebus monkey by Dowling and Ehinger 1978⁹ and Dowling, Ehinger and Florén 1980¹⁰. These investigators infer that most of the dopaminergic neurons in goldfish and Cebus monkey retinas are interplexiform. However, since no method of labeling is available that distinguishes between different kinds of dopaminergic neurons, it is at present impossible to exclude the possibility that there are also dopaminergic amacrine cells in the retinas of these animals. To further complicate the analysis of the dopaminergic neurons and their synaptic connections in the Cebus monkey, there may be an additional third kind of dopaminergic neuron called the pleomorph cell, present in the retina of this monkey⁵. This cell has not been seen in the Cynomolgus monkey, however.

When comparing the light- and electron microscopical appearance of the dopaminergic amacrine cell in the Cynomolgus monkey retina with earlier descriptions of Golgi-stained amacrine cells, it is found that this neuron most closely resembles the "smaller unistratified amacrine cell" described by Boycott and Dowling 1969 (page 154)²⁷. The processes of this unistratified cell ramify within one plane only of the inner plexiform layer, generally in the scleral half. In the paper of Boycott and Dowling there is a picture of a unistratified amacrine cell in the Rhesus monkey retina (Fig. 62, page 152) which has a very similar appearance to that of the dopaminergic amacrine cell in the Cynomolgus monkey. The amacrine cell pictured has its perikaryon in the innermost part of the inner nuclear layer and its processes ramify in the inner plexiform layer along the junction of the inner nuclear and inner plexiform layers. Boycott and Dowling do not describe any

processes extending from this particular amacrine cell into the inner nuclear layer, but in the picture mentioned, a process can be seen extending into the inner nuclear layer close to the stained perikaryon in the same way that dopaminergic processes are seen in Cynomolgus retinas processed according to the method of Falck and Hillarp. However, it is not clear from the picture nor the text of the Boycott and Dowling paper if this particular process is connected with the stained neuron in question. It is noteworthy that both the Rhesus and Cynomolgus monkeys are Old World monkeys.

Figure 11 is a summary diagram showing a dopaminergic amacrine neuron with its perikaryon in the innermost row of amacrine cell bodies in the inner nuclear layer and the vast majority of its processes limited to the outermost sublayer of the inner plexiform layer. One process is drawn partially surrounding another amacrine cell perikaryon. The dopaminergic processes make synapses onto both cell bodies and processes of other amacrine cells. One synapse is depicted from a non-dopaminergic amacrine cell process onto a varicosity of a dopaminergic process. There are no direct synaptic connections between the dopaminergic neuron and bipolar or ganglion cells.

One puzzle is the difficulty with which the input synapses onto dopaminergic processes are detected. The same difficulties were encountered when the synaptic connections of the dopaminergic neurons in the retina of the rabbit and the Cebus monkey were analyzed^{10,16}. In the rabbit the synaptic input from other amacrine neurons was found to be always onto the intervaricose segments of the dopaminergic cells. The chance of finding labeled organelles such as synaptic vesicles or mitochondria in the thin intervaricose segments is small and it is also hard to trace these intervaricose segments in serial sections. This may explain some of the difficulties in finding the input synapses from non-dop-

aminergic neurons in that animal. In the present study, the number of input synapses onto dopaminergic processes observed was also very small and generalized conclusions about the parts of neuronal processes involved cannot be made. Another reason why input synapses are so infrequently observed is that they tend to be inconspicuous with only three or four synaptic vesicles seen in the presynaptic process in any one section and very little in the way of membrane specialization at the presumed presynaptic site. However, the difference in the number of output and input synapses is substantial enough to warrant the conclusion that output synapses are far more common than input synapses.

The synaptic arrangement of the dopaminergic amacrine cells is similar to that of the dopaminergic neurons in the retina of the rabbit¹⁶ and that seen in the inner plexiform layer of goldfish and Cebus monkey^{8,9,10}. Thus, where investigated, the dopaminergic neurons in the inner plexiform layer have been found to contact only amacrine cells. Other kinds of amacrine cells, on the other hand, are known to have contacts with bipolar neurons and ganglion cells, as well as other amacrine cells.

From the time of Cajal it has been recognized that the amacrine cells of the retina can be divided into subclasses on a morphological basis. In recent years a multitude of neurotransmitters have been indicated for the amacrine neurons (acetylcholine, dopamine, an indole, amino acids and possibly peptides)^{3,28,29,30,31,32}. Thus a number of pharmacologically distinct subclasses of amacrine cells have emerged. Ultrastructural analysis has demonstrated that within one animal different pharmacological subclasses of amacrine neurons may have different morphology and different synaptic organization. In the rabbit retina, for instance, the dopaminergic neurons have their dendritic arborizations within three sublayers in the inner plexiform layer, but mainly within the outermost sublayer. They exchange synaptic information with other amacrine cells only¹⁶. The indoleamine-accu-

modulating neuronal processes, on the other hand, ramify chiefly in the innermost sublayer of the inner plexiform layer. The vast majority of their synaptic connections are formed with bipolar cells, generally in a reciprocal arrangement²³.

It is also known from electrophysiological studies that neurotransmitter substances such as acetylcholine, gamma-aminobutyric acid and glycine, their agonists and antagonists have different effects on the ganglion cells of the retina, and that these effects often vary considerably from species to species^{33,34,35,36,37,38}. As for dopamine, it has been shown to depress light-evoked excitation and to enhance light-evoked inhibition of ganglion cells in the cat retina after iontophoretical application or close intra-arterial injection^{39,40}. These responses are the same for on, off and on-off ganglion cells. In the isolated and perfused rabbit retina dopamine increases the activity of off ganglion cells and decreases the spontaneous and evoked activity of on and on-off ganglion cells⁴¹. An extensive study of the effects of dopamine on the retina of the goldfish suggests that the dopaminergic neurons (which are interplexiform in the goldfish) act on the luminosity-type cone horizontal cells and bipolar cells in the outer plexiform layer and the transient amacrine cells in the inner plexiform layer⁴². Dopamine decreases the light responsiveness of the horizontal cells suggesting that the dopaminergic cells regulate lateral inhibitory effects and center-surround antagonism in the outer plexiform layer. The above results indicate several functions for dopaminergic neurons in the retina. However, much more detailed investigations are needed before the function of the various types of dopaminergic neurons throughout the animal series is clear.

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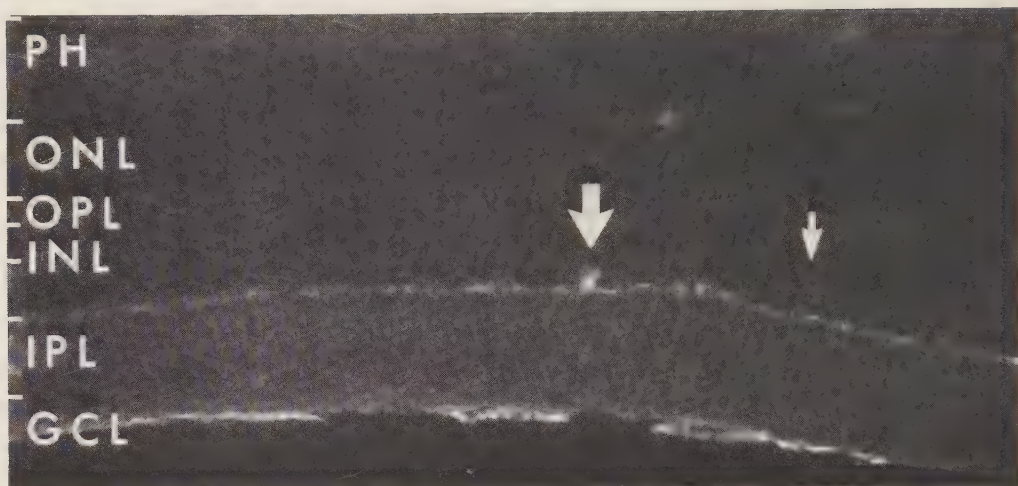


Fig. 1a.

Fluorescence micrograph of a control retina of a Cynomolgus monkey, processed according to the Falck and Hillarp method. A fluorescent dopaminergic cell body (large arrow) is seen in the innermost part of the inner nuclear layer. Fluorescent processes arborize at the junction of the inner nuclear and inner plexiform layers. One fluorescent process is seen to extend between the innermost perikarya of the inner nuclear layer (small arrow). PH = photoreceptors, ONL = outer nuclear layer, OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer. X 265.

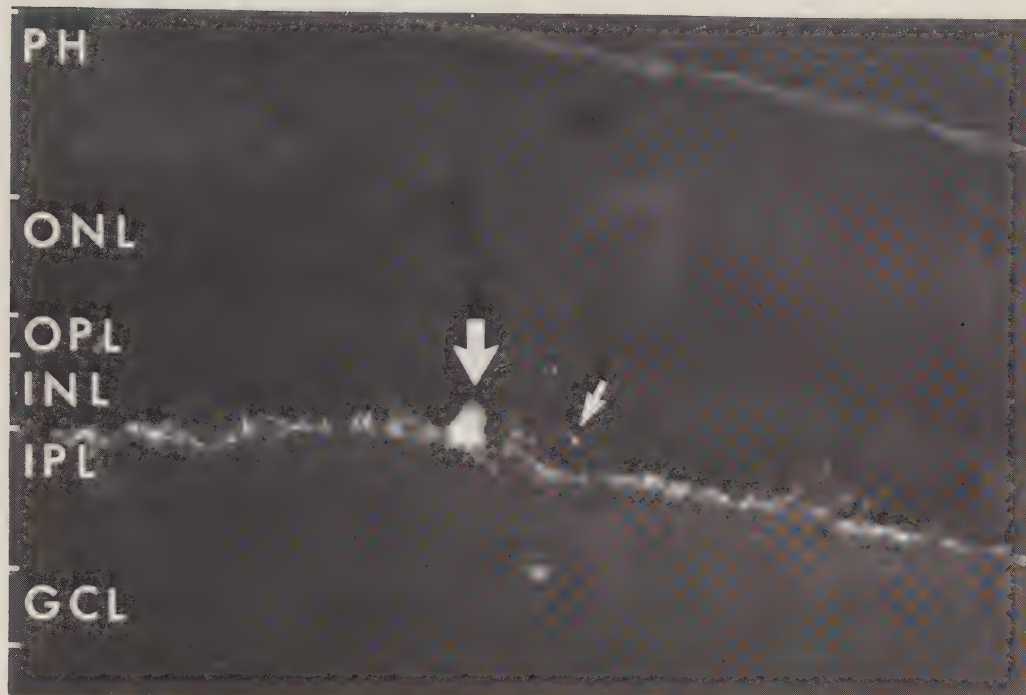


Fig. 1b.

Cynomolgus monkey retina processed according to the Falck and Hillarp method after intravitreal injection of 5,6-dihydroxytryptamine. There is a fluorescent perikaryon in the innermost row of amacrine cell bodies in the inner nuclear layer (large arrow). The fluorescent neuronal processes ramify at the junction of the inner nuclear and inner plexiform layers with occasional processes extending between and around the innermost non-fluorescent cell bodies in the inner nuclear layer (small arrow). The distribution of fluorescent perikarya and processes is similar to the one seen in the control retina of fig. 1a. There is no evidence of uptake of 5,6-dihydroxytryptamine into any other cell population in the retina than the dopaminergic cells. Fluorescence micrograph. X 430.

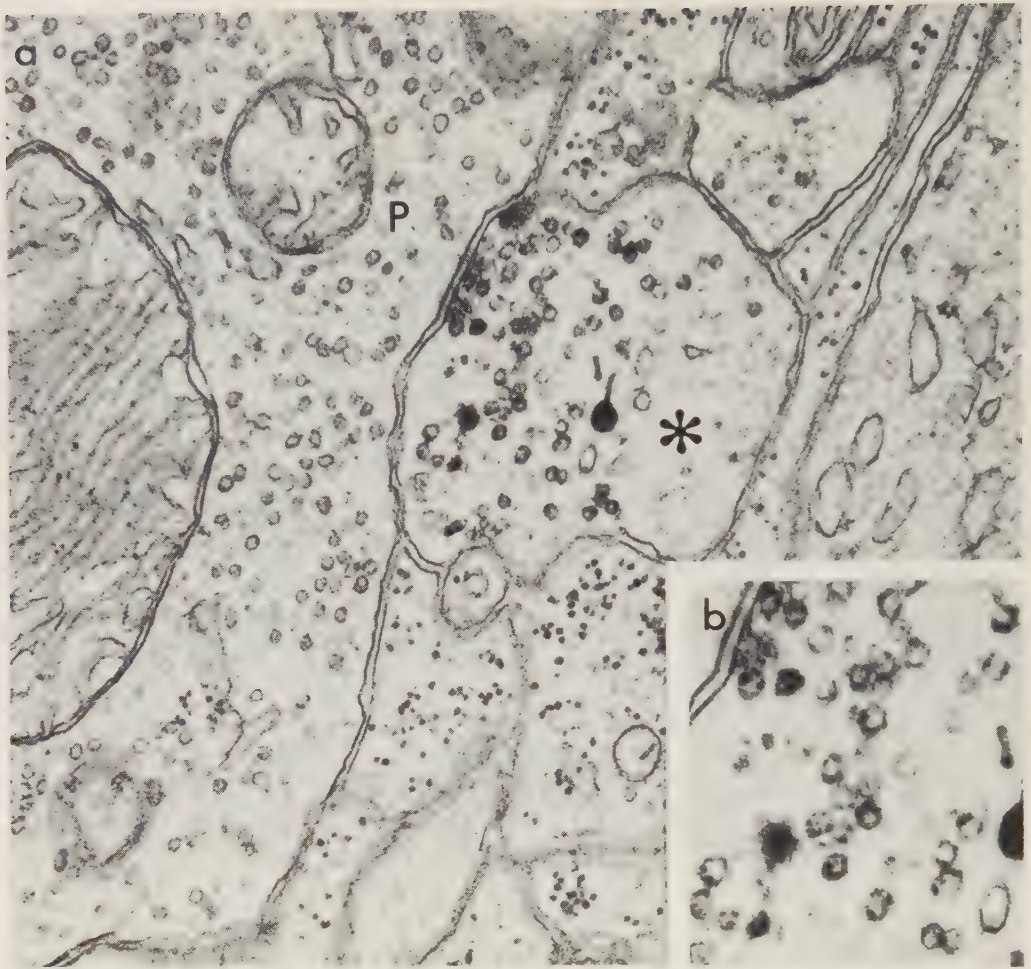


Fig. 2a and b.

Electron micrograph of a labeled dopaminergic process (asterisk) with an output synapse onto an unidentified neuronal process (P) in the outermost sublayer of the inner plexiform layer. There is a presynaptic cluster of small synaptic vesicles in the labeled process. On the pre- and postsynaptic membranes as well as in the synaptic cleft there is electron dense material. Many of the small synaptic vesicles in the labeled process have increased electron density of their membranes with eccentric spots of dense material. A few of them are flattened in shape. A large synaptic vesicle, completely electron dense, is seen with a thin process extending out. There are also a few vesicular structures of intermediate size. In b the small synaptic vesicles are shown in higher magnification. OsO₄ fixation. a X 52,000, b X 92,000.

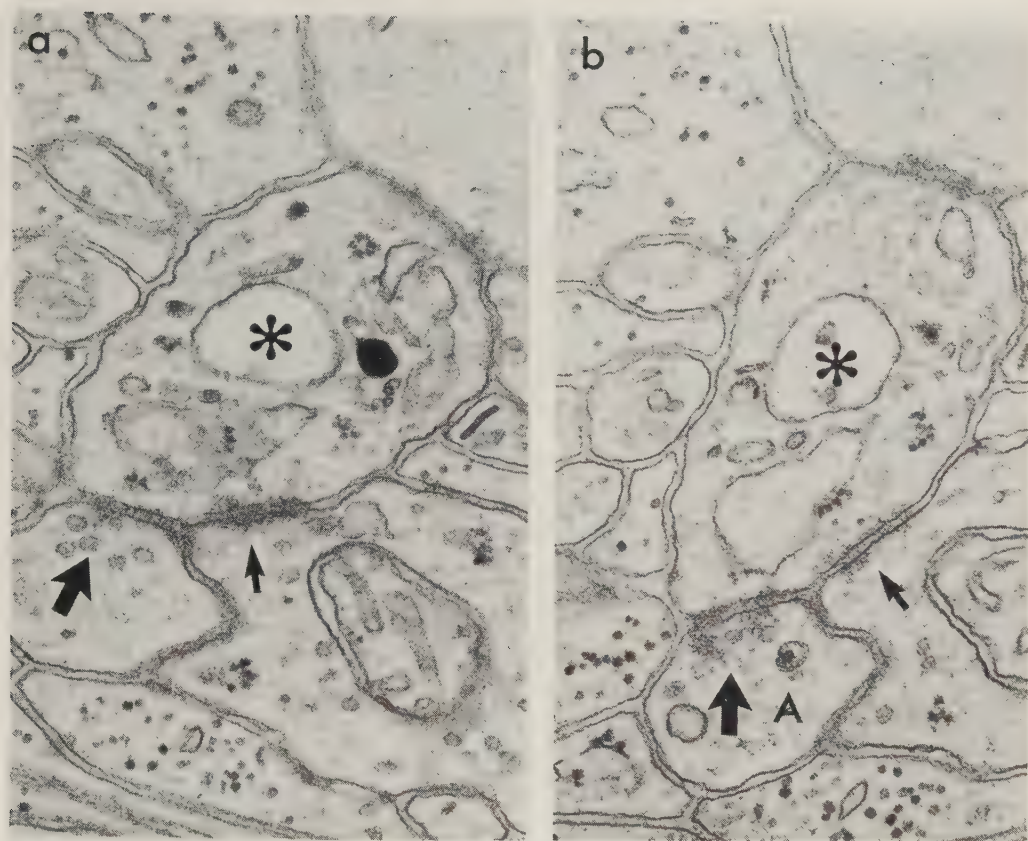


Fig. 3a and b.

Synapse from an amacrine process (A) onto a labeled varicosity (asterisk). The presynaptic vesicles are few (large arrow) and the pre- and postsynaptic membrane densities inconspicuous. The electron dense material in the synaptic cleft is clearly visible. A desmosome-like junction between the labeled varicosity and an adjacent neuronal process is also seen (small arrow). There are fairly symmetrical electron dense depositions on the junctional cell membranes and dense material in the intercellular cleft. No vesicles are seen in the vicinity of this junction. The labeled varicosity contains a large synaptic vesicle filled with dense material to its limiting membrane and a few labeled small vesicles. In b the same input synapse is seen in an adjacent section. There are no clearly labeled cell structures in the dopaminergic process. Outer sublayer of the inner plexiform layer. OsO₄ fixation. a and b X 54,000.

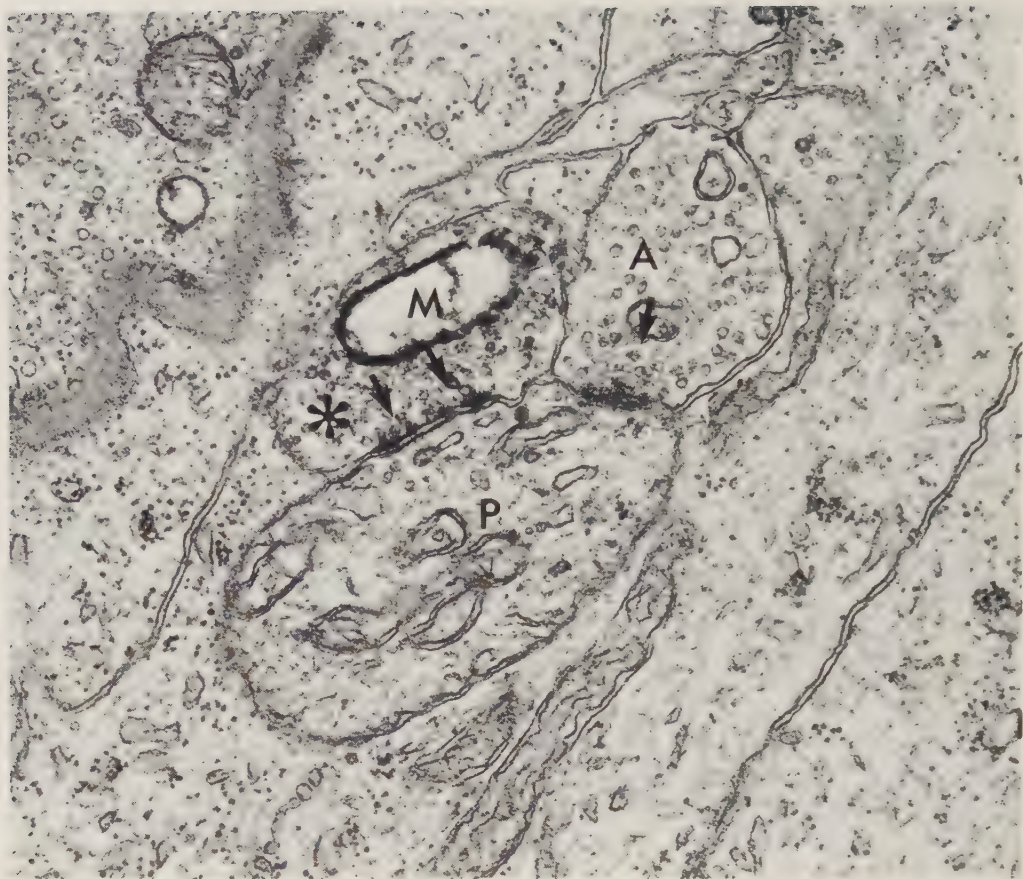


Fig. 4.

One labeled dopaminergic process (asterisk) and one unlabeled amacrine process (A) with output synapses (arrows) onto the same unlabeled varicosity (P) at the junction of the inner nuclear and inner plexiform layers. The labeled process contains a swollen mitochondrion with increased density of the outer membrane (M) and some labeled small synaptic vesicles. The synapses are of the conventional kind with a presynaptic cluster of synaptic vesicles, dense depositions on the pre- and postsynaptic membranes as well as increased density of the membranes themselves, and electron dense material in the synaptic cleft. The synaptic cleft between the dopaminergic process and the unlabeled varicosity appears to be slightly widened. In the upper left hand corner of the electron micrograph the basal membrane of a retinal capillary is seen. OsO₄ fixation. X 46,000.

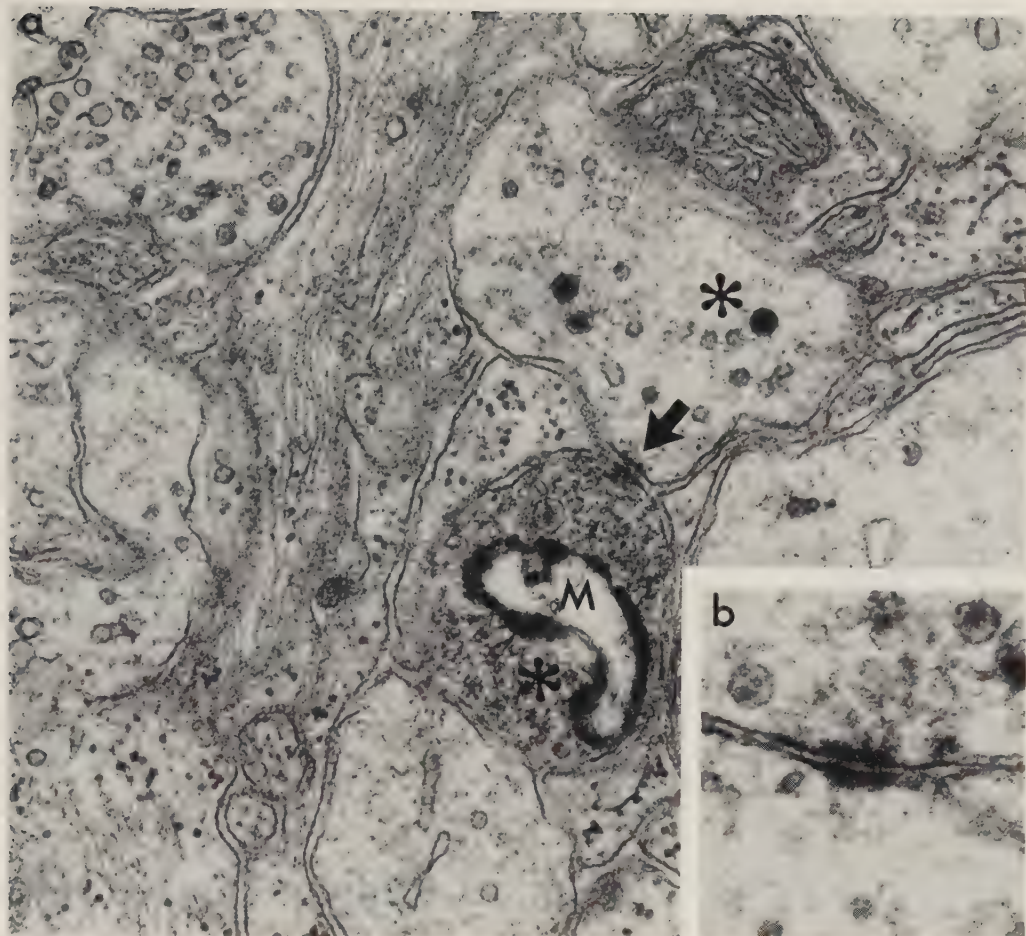


Fig 5a.

Two juxtapsed labeled processes (asterisks) in the outermost sublayer of the inner plexiform layer. One labeled process is filled with small synaptic vesicles and has a distorted labeled mitochondrion (M). The other dopaminergic process displays only a few labeled small synaptic vesicles and three large vesicles whose central cores have a varying degree of electron density. The processes are connected by a desmosome-like junction (arrow). In this region there is increased density of the cell membranes, dense depositions on the cell membranes and electron dense material in the intercellular space. OsO₄ fixation. X 55,000.

Fig. 5b.

A high magnification electron micrograph of another desmosome-like junction between two labeled processes. Symmetrical depositions of electron dense material on the cell membranes are seen as well as dense material in the intercellular space. OsO₄ fixation. X 95,000.

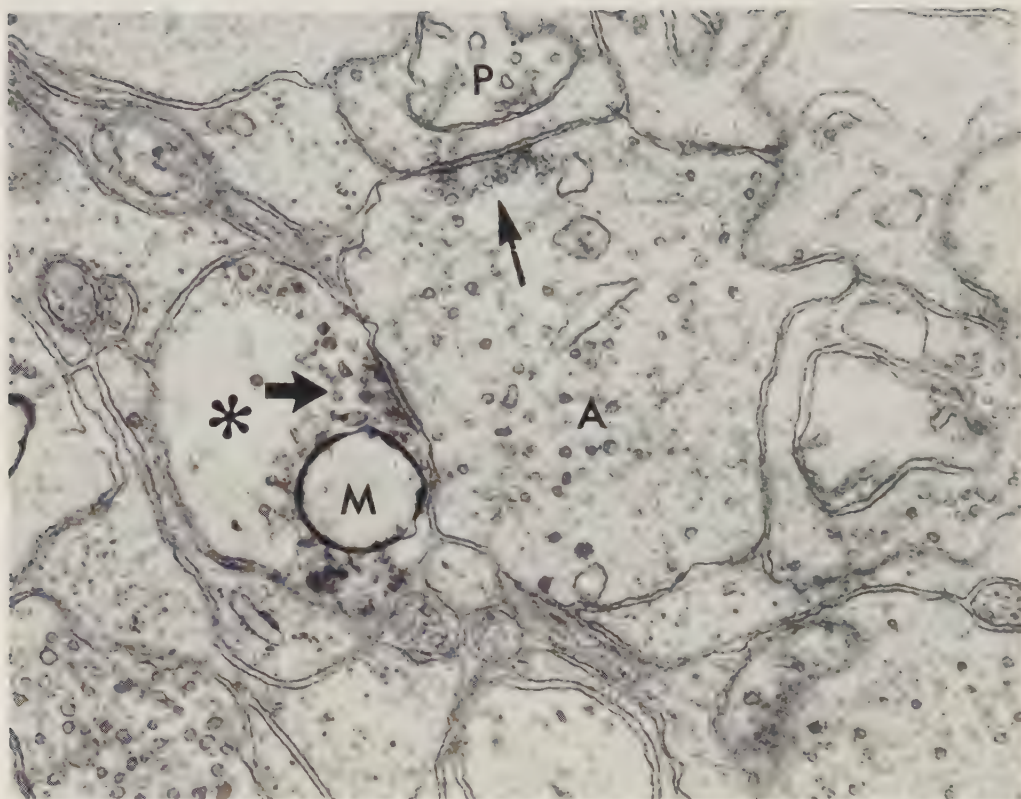


Fig. 6.

Synapse (large arrow) from a dopaminergic process (asterisk) onto an amacrine cell varicosity (A) which in turn has an output synapse (small arrow) onto an unlabeled process (P). The labeled varicosity appears slightly swollen with a part of it void of cell organelles. A swollen mitochondrion (M) is also seen. Part of another labeled process is shown to the left. OsO₄ fixation. X 61,000.

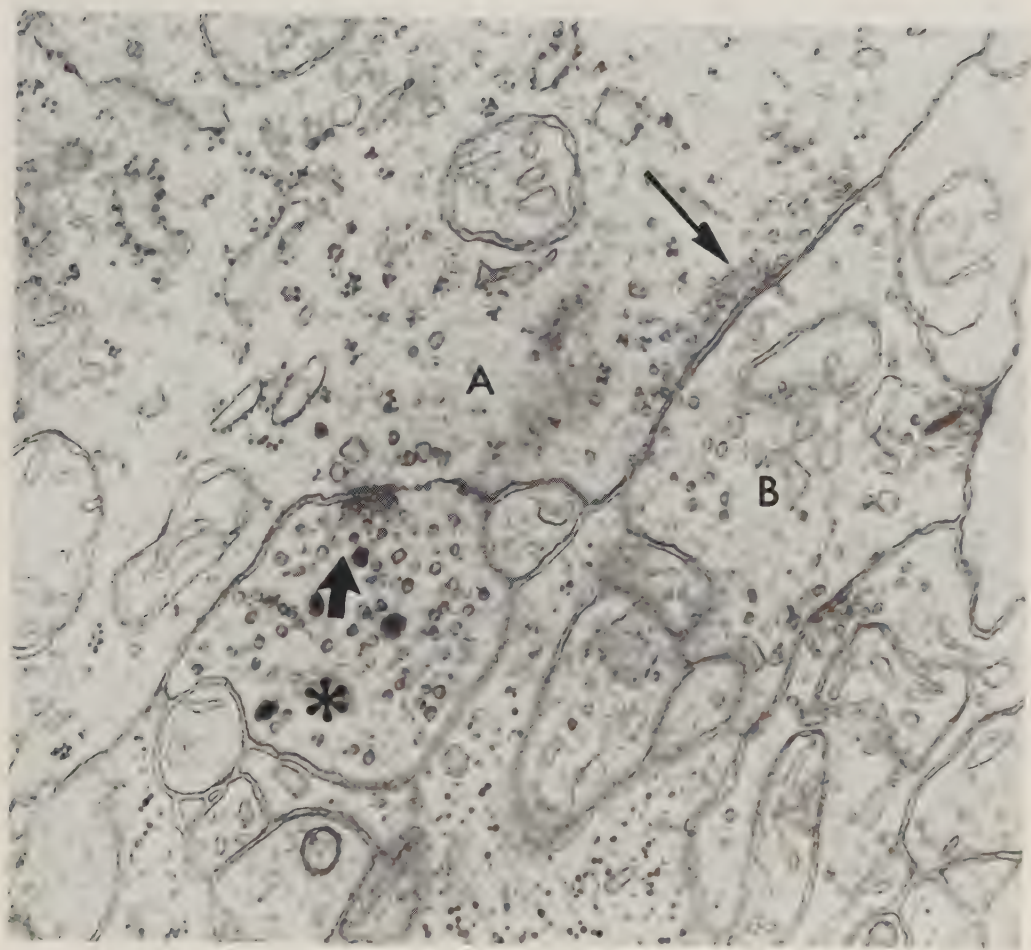


Fig. 7.

Serial synapse with an output synapse (large arrow) from a dopaminergic varicosity (asterisk) onto an amacrine cell body (A) which makes synaptic contact (small arrow) onto a bipolar terminal (B). The bipolar cell has a ribbon synapse onto two other neuronal processes in the dyad arrangement. OsO₄ fixation. X 36,000.

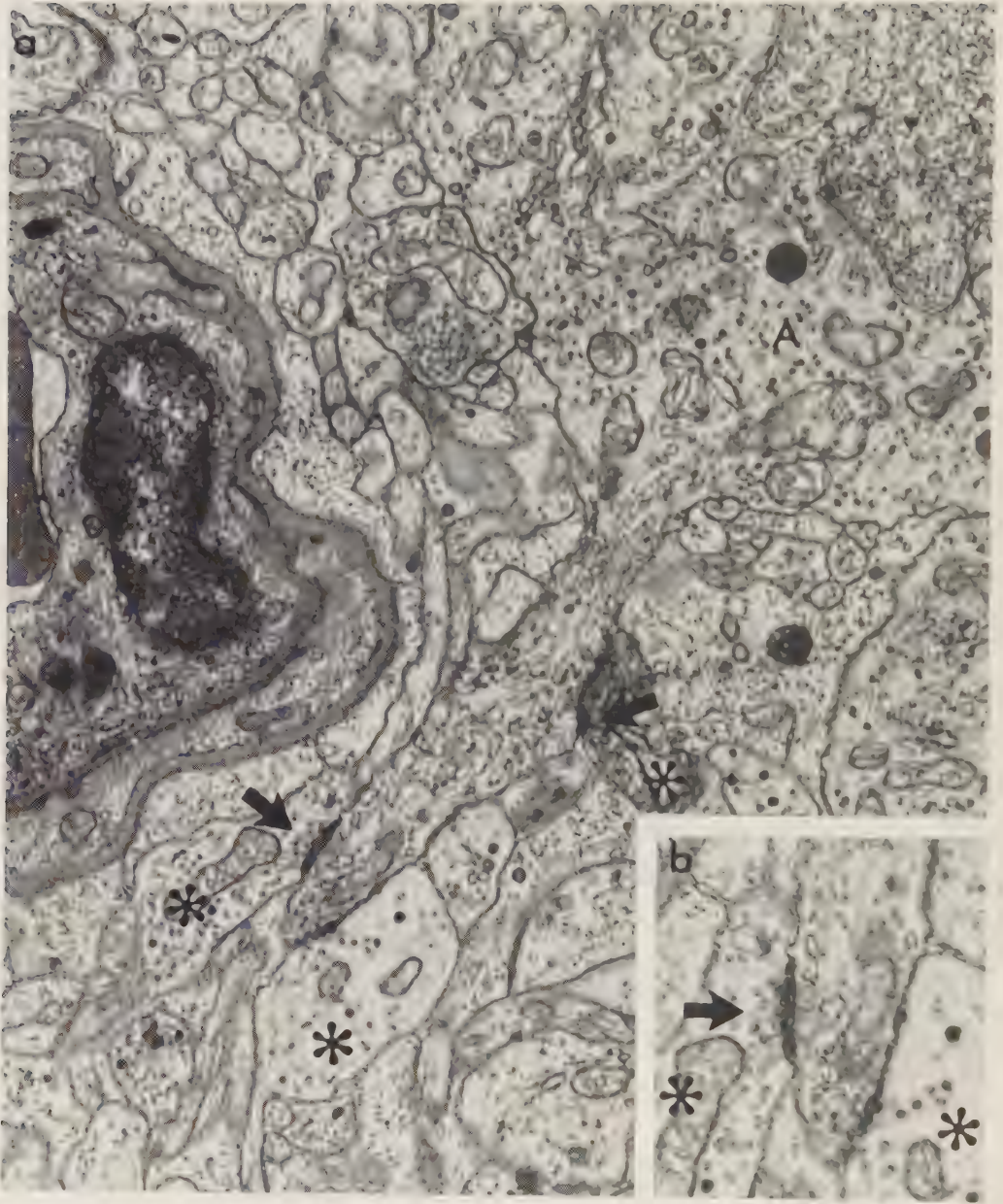


Fig. 9a and b.

Amacrine cell body (A) in the inner nuclear layer with a process extending into the inner plexiform layer. Three dopaminergic varicosities are juxtaposed to this amacrine cell process (asterisks). Two of the dopaminergic varicosities have output synapses onto the process (arrows). There are two more labeled processes in the electron micrograph and to the left in the picture there is a retinal capillary. In inset b one of the synapses is seen in higher magnification. OsO₄ fixation. a X 25,000, b X 36,000.

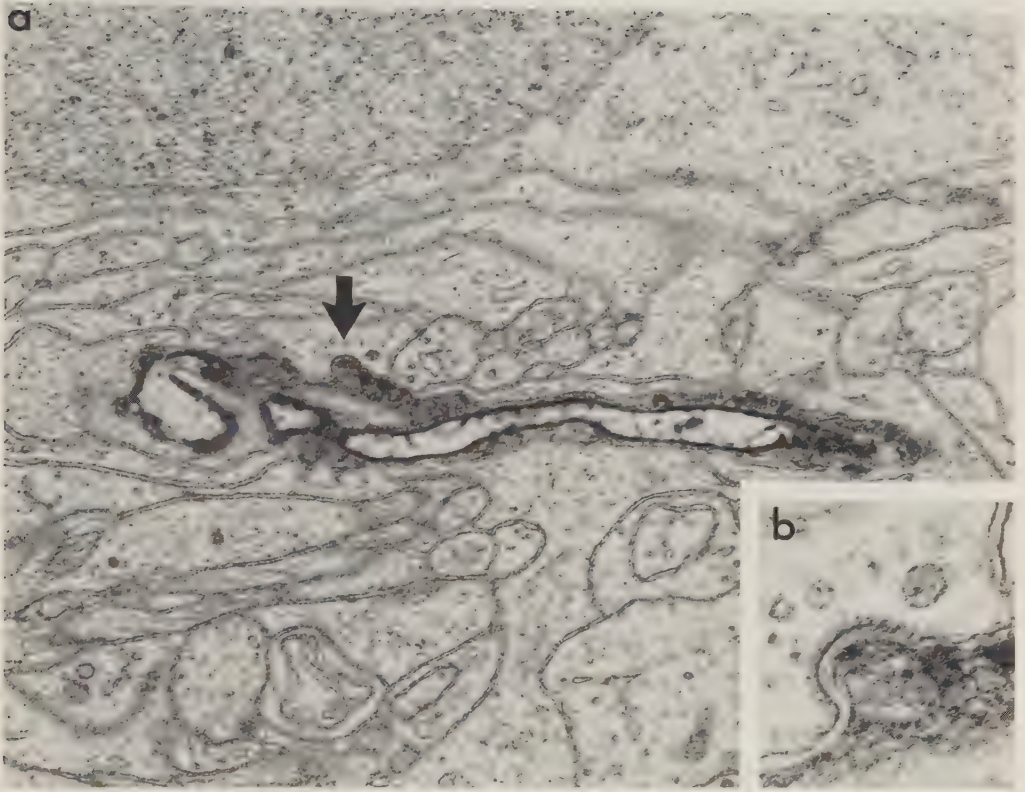


Fig. 9a and b.

A labeled dopaminergic process at the junction of the inner nuclear and inner plexiform layers. The process is shrunken and filled with small synaptic vesicles of increased electron density and swollen distorted mitochondria. A junction between the labeled process and an unidentified neighboring process is seen at arrow and in b in higher magnification. OsO_4 fixation. a X 29,000, b X 110,000.

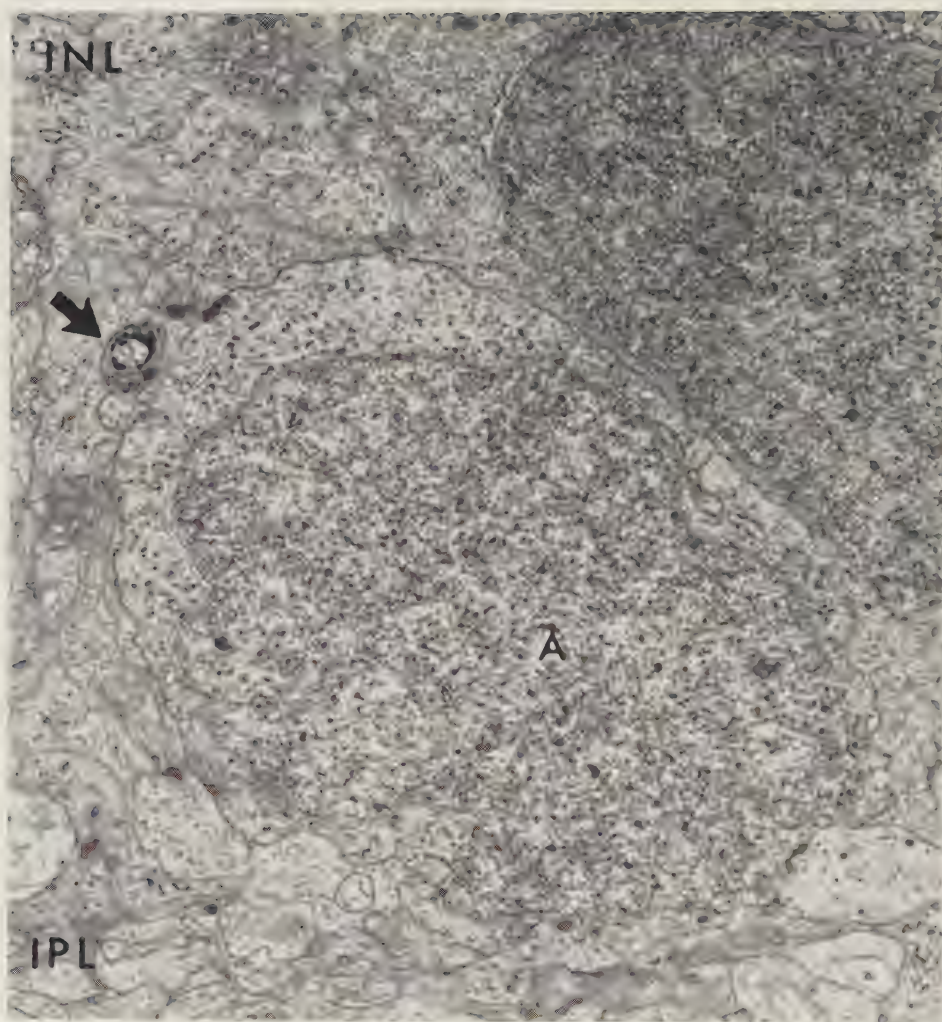


Fig. 10.

Labelled dopaminergic process (arrow) on the outside of an amacrine cell perikaryon (A) in the innermost row of cell bodies in the inner nuclear layer. OsO₄ fixation. X 14,000.

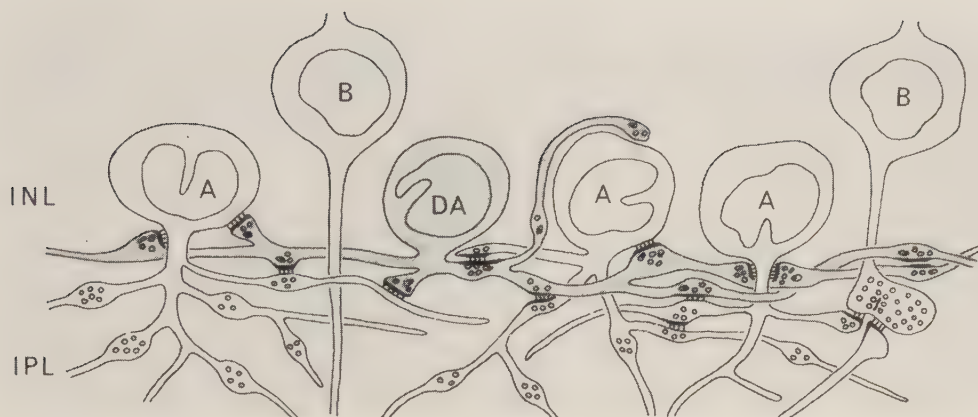


Fig. 11.

Summary diagram of a dopaminergic neuron and its synaptic connections in the Cynomolgus monkey retina. Its perikaryon is situated in the innermost row of amacrine cell bodies in the inner nuclear layer. Its dendrites arborize almost exclusively in the outermost sublayer of the inner plexiform layer. One process extends into the inner nuclear layer to the outside of an amacrine cell perikaryon. The dopaminergic processes make synaptic contacts onto the amacrine cell bodies and their processes as they emerge from the cell bodies at the junction of the inner nuclear and inner plexiform layers. They also have numerous output synapses onto the more distal varicosities and intervaricose segments of amacrine neurons. A dopaminergic output synapse forming part of a serial synapse with two other non-dopaminergic amacrine cells is depicted. The dopaminergic neuron receives synaptic input from a non-dopaminergic amacrine process. The synaptic structures of this connection are discrete. A desmosome-like junction is shown between two dopaminergic processes. No direct synaptic contacts between the dopaminergic neuron and bipolar or ganglion cells are found. Thus, the dopaminergic cells form a class of interamacrine neurons.

ULTRASTRUCTURE AND SYNAPSES OF THE (^3H)-DOPAMINE-ACCUMULATING
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SUMMARY

The dopaminergic neurons in the retina of the adult rabbit have previously been studied in the light microscope using the Falck and Hillarp technique for fluorescence microscopy of structures containing biogenic monoamines and using an autoradiographic technique with (^3H)-dopamine. Their synaptic connections have been investigated in the electron microscope after labeling with 5,6-dihydroxytryptamine. This labeling method has the advantage of high resolution, but the disadvantage of a certain variability in the labeling as well as of obscuring the normal cytological structure of the labeled neurons. The 5,6-dihydroxytryptamine labeling method also requires the prior destruction of the otherwise interfering indoleamine-accumulating neurons. In this study, an autoradiographic technique with a monoamine oxidase inhibitor and (^3H)-dopamine has been used to label the dopaminergic neurons. The autoradiographic technique preserves the normal ultrastructure of the dopaminergic neurons and does not result in any significant labeling of the indoleamine-accumulating neurons.

The investigation confirms that the (^3H)-dopamine-accumulating neurons are amacrine cells with large cell bodies situated in the innermost row of the inner nuclear layer. Their processes ramify in three bands in the inner plexiform layer. The outermost band is the densest one. In addition to the usual neuronal organelles the cytoplasm of the (^3H)-dopamine-accumulating cell bodies and their processes characteristically contain large dense-cored vesicles of 90-160 nm diameter.

The (^3H)-dopamine-accumulating processes have output synapses only to other amacrine cells, onto both their cell bodies and processes. Input synapses from other amacrine cells to the (^3H)-dopamine-accumulating processes are found onto their intervaricose segments and small varicosities. The (^3H)-dopamine-accumulating neurons form interamacrine connections only.

KEY WORDS: Retina - (³H)-dopamine-accumulating - Ultrastructure - Synapses - Rabbit

INTRODUCTION

Dopaminergic retinal neurons have been known for almost two decades and have been reported in a great number of animal species (for a review, see Ehinger, 1982). They were first demonstrated in retinæ processed according to the Falck and Hillarp technique for fluorescence microscopy of structures containing biogenic monoamines. In many animal species, including humans, the dopaminergic neurons were found to be a subclass of amacrine cells: they have their cell bodies in the innermost cell row of the inner nuclear layer and their processes are confined to the inner plexiform layer. In addition, it was discovered that in some animals, such as New World monkeys and teleost fish, the dopaminergic neurons form a separate type of retinal neurons, called the interplexiform cells. These cells have their cell bodies in the innermost part of the inner nuclear layer like the dopaminergic amacrine cells of other species, but their processes ramify widely in both the inner and outer plexiform layers.

The dopaminergic retinal neurons can also be visualized in the electron microscope using a labeling technique with 5,6-dihydroxytryptamine. After intravitreal injection, this indoleamine is taken up by the dopaminergic neurons and causes characteristic ultrastructural changes in them. These changes consist of increased density of large dense-cored synaptic vesicles, eccentric dense spots in small synaptic vesicles and increased density and distortion of mitochondria and other cell organelles as well as of the normal cytoplasmic structure. Thus, the ultrastructural changes make it possible to identify the dopaminergic neurons, but the changes also prevent the study of the normal ultrastructure of these neurons.

5,6-dihydroxytryptamine is also taken up by a population of indoleamine-accumulating retinal neurons present in most vertebrates (Ehinger and Florén, 1980; Ehinger, Hansson and Tornqvist, 1982). Furthermore, 5,6-dihydroxytryptamine causes similar ultrastructural changes in both dopaminergic and indoleamine-accumulating neurons. Consequently, in order to analyze the synaptic contacts made by those two populations of neurons, the processes of one or the other population have to be destroyed. Methods for the selective destruction of dopaminergic or indoleamine-accumulating processes are available, using repeated intravitreal injections of 6-hydroxydopamine and 5,7-dihydroxytryptamine respectively (Ehinger and Nordenfelt, 1977; Ehinger and Florén, 1978a). Neither of these methods will, however, completely destroy all the processes of either kind without inducing non-specific damage to the retina.

The labeling method with 5,6-dihydroxytryptamine has been successfully used in several investigations mapping the synaptic organization of the dopaminergic and indoleamine-accumulating neurons in mammals (Dowling and Ehinger, 1975, 1978a; Ehinger and Holmgren, 1979; Dowling, Ehinger and Florén, 1980; Holmgren, 1982; Holmgren-Taylor, 1982a), fish (Dowling and Ehinger, 1975, 1978b; Holmgren-Taylor 1982b) and amphibians (Adolph, Dowling and Ehinger, 1980). In the rabbit retina the dopaminergic neurons were found to be amacrine cells with synaptic contacts only with other amacrine cells (Dowling and Ehinger, 1978a). Because of the disadvantages inherent in the labeling method with 5,6-dihydroxytryptamine, it was considered desirable to repeat the investigation using a labeling method which would neither disrupt the normal cytoarchitecture of the dopaminergic processes nor require the prior destruction of indoleamine-accumulating processes with a neurotoxic drug. The dopaminergic neurons have a specific and selective system for re-uptake of their neurotransmitter (Kramer, 1971; Ehinger and Florén, 1978b; Sarthy and Lam, 1979). This

uptake system can be used to label the dopaminergic neurons with (^3H)-dopamine for autoradiography (Ehinger and Falck, 1971; Kramer, Potts and Mangnall, 1971; Lam, Fung, and Kong, 1981), and it has been possible to find a labeling procedure which does not result in significant labeling of the indoleamine-accumulating neurons. Although the resolution of any autoradiographic labeling method is less than the size of the smallest nerve cell process in the retina and far less than the best resolution obtainable in the electron microscope, it is sufficient for analysis of the ultra-structure and the synapses made by the dopaminergic neurons. The observations made in this investigation confirm the synaptic organization for the dopaminergic neurons in the retina of the rabbit earlier described and reveal some interesting cytological characteristics of these neurons.

MATERIALS AND METHODS

Two sets of experimental eyes were used. For the first set, two young adult albino rabbits (1.5 - 2 kg body weight) were injected intraperitoneally with 50 mg/kg body weight of Pargyline, a monoamine oxidase inhibitor; followed 1/2 hour later by an intravitreal injection of 50 μCi of (^3H)-dopamine. The eyes were enucleated 4 hours later. Two eyes were treated in this way.

For the second set of experimental eyes, which also served as a control for the first set of eyes, six young adult albino rabbits were injected intraperitoneally with 50 mg/kg body weight of Pargyline and 1/2 hour later intravitreally with 50 μg of 5,7-dihydroxytryptamine. This procedure was repeated on the following day. After one week, when the processes of the indoleamine-accumulating neurons had degenerated (Ehinger and Florén, 1978a), the rabbits were again given 50 mg/kg body weight of Pargyline intraperitoneally and then, after 1/2 hour, 50 μCi of (^3H)-dopamine in an intravitreal injection. This experimental set consisted of six eyes.

Two control eyes were subjected to the 5,7-dihydroxytryptamine treatment only. All injections were given with a Hamilton precision syringe. 5,7-dihydroxytryptamine was dissolved in 0.9% NaCl containing 1 mg/ml ascorbic acid as an antioxidant. (³H)-dopamine was injected as delivered as the hydrochloride in an aqueous solution.

After enucleation, the eyes were incised at the ora serrata, and the anterior segments were cut away and discarded. A few small pieces of both the experimental and control retinæ treated with 5,7-dihydroxytryptamine were processed for fluorescence microscopy of biogenic monoamines with the Falck and Hillarp method as described by Björklund, Falck and Owman, 1972, in order to check that the removal of the indoleamine-accumulating processes had not also affected the dopaminergic ones. These retinal sections were viewed in the fluorescence microscope with a barrier filter with its 50% cut-off edge at about 500 nm.

The rest of the posterior segments were then fixed for 1 1/2 hours in ice cold 2.5% glutaraldehyde in 65 mM cacodylate buffer (pH 7.8) with 4.5 mM CaCl₂. After a brief wash in 67 mM cacodylate buffer with 0.13 M sucrose, the posterior segments were postfixed in 2% OsO₄ in veronal acetate buffer (pH 7.8) containing 28 mM barbital sodium, 28 mM sodium acetate, 66 mM sucrose and 1.8 mM CaCl₂ for 1 hour in an ice bath and for another 1/2 hour at room temperature. The tissue was then washed in double strength veronal acetate buffer, dehydrated in graded alcohols and embedded in Epon 812.

For light microscopy autoradiography, 4 µm retinal sections were cut and placed on glass slides, which were then coated with either Kodak AR-10 emulsion by the stripping technique or Ilford L-4 emulsion, dilution 1:1, by the dipping technique (see, for example, Rogers, 1979). The emulsion-coated slides were exposed for two weeks, then developed in undiluted D-19 for the Kodak emulsion and in Dektol with a 1:1 dilution for the Ilford emul-

sion. Fixation was carried out in 30% sodium thiosulfate. The sections were analyzed in a light microscope using phase contrast optics. The frequency of radioactive cell bodies was counted and their sizes were measured using an ocular micrometer.

Ultrathin sections for electron microscopy were transferred to collodion-coated glass slides using a silver loop. After drying, the sections were stained on the glass slides with 2% uranyl acetate in 47.5% ethanol for 20 min and Reynold's lead citrate solution for 5 min. The glass slides were subsequently carbon coated, dipped in Ilford L-4 emulsion, dilution 1:4, and exposed for about two weeks. They were developed in Phenidone (Boyenval and Fischer, 1976) for 1 min at 15°C and fixed in 30% sodium thiosulfate. The collodion membranes with the sections were floated off the glass slides onto a surface of distilled water, and both Formvar-coated and uncoated bar- and slot-grids were placed on top of the sections. After drying, the grids were cut out from the collodion membrane, and the collodion membrane on the grids was thinned in amyl acetate for either 3 or 15 min, depending on whether the grids were Formvar-coated or not (the longer time for coated grids). The sections were viewed in a JEOL 100B electron microscope.

2,5,6-(³H)-dopamine, 3,4-Dihydroxy(ring-2,5,6-³H)phenylethylamine hydrochloride, with the specific activity 366 GBq/mmol = 9.9 Ci/mol, was obtained in an aqueous solution from Amersham. 5,7-dihydroxytryptamine was obtained from Regis Chemical Co., Morton Grove, Illinois, U.S.A., and Pargyline from Sigma Chemical Co., St. Louis, Missouri, U.S.A. All weights given refer to the salts.

RESULTS

Light microscopy

The radioactive label was concentrated in cell bodies, mainly in the inner nuclear layer, and in three bands in the inner plexiform layer (Fig. 1). The density of labeled cell bodies was

0.3 $\pm$ 0.05 (s.e.m.) per mm retina; 103 cells counted in 4 μ m sections of five randomly obtained pieces of experimental retinae. The majority of the labeled cell bodies were found along the inner border of the inner nuclear layer, among the amacrine cell bodies. Labeled cell bodies were also encountered in the ganglion cell layer, but much less frequently than in the inner nuclear layer. Among a total of 3027 cell bodies in the ganglion cell layer, counted in four randomly obtained pieces of retina, four labeled cell bodies were observed, constituting about 0.1% of the total number of cell bodies. Rarely were labeled cell bodies detected in the inner plexiform layer. The number of such cell bodies was too small to give any meaningful figure of frequency.

The labeled cell bodies in the inner nuclear layer were generally larger than the majority of unlabeled cell bodies in the innermost cell row of that layer. The mean diameter of 28 measured labeled cell bodies in the inner nuclear layer was 9.8 $\pm$ 0.38 μ m (s.e.m.), whereas the mean diameter of 63 adjacent unlabeled cell bodies in the inner nuclear layer was 8.9 $\pm$ 0.27 μ m. The difference in diameter is statistically significant ($p < 0.01$) in Student's two tailed t-test. In the ganglion cell layer, the labeled cell bodies were usually of the same size as those of the inner nuclear layer, but the ones in the inner plexiform layer appeared to be smaller.

Three bands of radioactivity were seen in the outermost, middle and innermost sublaminae of the inner plexiform layer. The outermost band, close to the border between the inner nuclear and inner plexiform layers, was the densest one. The middle band was weaker, and the innermost band was weakest. The labeled cell bodies of the inner nuclear layer were sometimes seen to send stout processes to either of the three bands of label in the inner plexiform layer.

No radioactive cell bodies or processes were observed further outwards than the cell bodies in the inner nuclear layer.

The distribution of labeled cell bodies and processes was the same in both the 5,7-dihydroxytryptamine and (^3H)-dopamine injected eyes as well as the eyes injected with (^3H)-dopamine only. The background level of radioactivity was consistently low in both types of experimental retinae.

The experimental and control retinae, which were treated with 5,7-dihydroxytryptamine, showed green fluorescence in three bands in the inner plexiform layer, corresponding exactly to the three bands of radioactive label seen in the experimental retinae injected only with (^3H)-dopamine. There was consequently no evidence that the dopaminergic neurons had been adversely affected by the treatment with 5,7-dihydroxytryptamine. All experimental and control animals received Pargyline prior to the intraocular injections.

Electron Microscopy

Distribution of label

The distribution of labeled cell bodies and processes in both sets of experimental retinae was similar to that seen in the light microscope. The density of silver grains was high in cell bodies, mainly in the innermost cell row of the inner nuclear layer. The grains were randomly distributed in the nuclei of labeled cell bodies, whereas they appeared to cluster around large dense-cored vesicles in the cytoplasm (Fig. 2). They were less frequent over the rough endoplasmic reticulum in the cytoplasm.

Clusters of grains were seen in three bands in the inner plexiform layer; the outermost one being the densest. The distribution of grains in the inner plexiform layer is given in Table I. It is noteworthy that almost half of the total number of silver grains were located in sublamina 1. The clusters of grains were seen both in association with varicosities of small to interme-

diate size (0.4 - 1.0 μ m) as well as with thin processes (smaller than 0.4 μ m). Very thin processes were often seen in bundles in the outermost sublayer of the inner plexiform layer, where they were surrounded by glial cells. The size of these processes was less than the resolution of the autoradiograms, and it was therefore impossible to identify exactly the labeled process or processes in a single random section. Processes smaller than 0.3 μ m were therefore judged as labeled only if they were clearly associated with grains in three or more consecutive sections. The labeling was very consistent through many sections; clusters of grains over a varicosity were traced in up to eight sections. It was also discovered that the joint occurrence of two or more grains in a varicosity and one or several large dense-cored vesicles (to be described in detail later) in that varicosity was an unequivocal indicator of a labeled process. This relationship was determined through analysis of several series of consecutive sections. The silver grains typically clustered around these large, dense-cored vesicles in the labeled processes (Fig. 3).

Ultrastructure of labeled cell bodies

Six labeled cell bodies were observed in the electron microscope (Fig. 2). They were clearly larger than most surrounding cells, and among the largest in the innermost cell row of the inner nuclear layer. The nuclei of the labeled cell bodies were also larger than those of the adjacent cell bodies, and they showed the indentations considered characteristic of amacrine cell nuclei. The chromatin pattern was rather finely granular, giving the nuclei a more translucent appearance than that of most unlabeled nuclei. The nucleoli were usually prominent in the labeled nuclei, and other intranuclear structures, in particular intranuclear rodlets, were also observed.

The labeled cell bodies had a comparatively wide rim of cytoplasm surrounding the nucleus. The cytoplasm appeared less dense than that of unlabeled cells in the inner nuclear layer.

This appearance was at least in part due to an abundance of rough endoplasmic reticulum. The cytoplasm also contained a comparatively large number of mitochondria. Golgi complexes and irregularly shaped lysosomes were frequently observed.

Large dense-cored vesicles were often seen dispersed in the cytoplasm (Fig. 3). Their diameter was about 90-160 nm. The vast majority of them had a characteristically large, homogenous, dense, central core, which left only a narrow space of about 5-12 nm between the core and the outer membrane. Occasionally, a large dense-cored vesicle with a smaller central core was seen in the cytoplasm of the labeled cell bodies. Both types of vesicles were also seen in unlabeled cells, but the ones with a large core were always more numerous in labeled cells, whereas the other type was more common in the unlabeled cells (Fig. 4).

Ultrastructure of labeled processes

When series of sections were analyzed, the labeled processes were seen to consist of thin intervaricose segments alternating with small to medium sized varicosities (0.4 - 1.0 μ m). The intervaricose segments contained microtubules as well as occasionally small synaptic vesicles and/or 90-160 nm vesicles with a large dense core (Fig. 4). The varicosities were usually filled with small, clear synaptic vesicles of 45-55 nm diameter. The large dense-cored vesicles were apparent in the labeled varicosities of most random sections and always found in series of sections (Fig. 3). The varicosities also contained mitochondria and occasionally microtubules.

Synapses

The labeled processes were seen to have output synapses of the conventional kind with an aggregation of small synaptic vesicles close to the presynaptic membrane and fibrillar dense material on the pre- and postsynaptic membranes as well as in the synaptic cleft (Figs. 5,6). In the inner plexiform layer, amacrine cells and interplexiform cells are known to have this kind of

synapses (Dowling and Boycott, 1966; Dowling and Ehinger, 1975, 1978b; Oyster and Takahashi, 1977; Kolb and West, 1977; Nakamura, McGuire and Sterling, 1980; Dowling et al., 1980). The vesicles with large dense cores did not appear to be associated with the synaptic structures although they frequently occurred in varicosities with synapses.

The synaptic contacts made by a sample population of labeled processes are shown in Table II. It is clear that the number of both output and input synapses in the inner plexiform layer paralleled the distribution of labeled processes in that layer. The (^3H)-dopamine-accumulating processes were found to have output synapses to only unlabeled amacrine cells. The vast majority of these synapses were onto amacrine cell varicosities (and possibly a few varicosities of interplexiform cells), identified by the presence of either a conventional synapse or a moderate number of unevenly distributed small synaptic vesicles (Fig. 5; Dowling and Boycott, 1966). In a few instances, the labeled processes were observed to have synapses onto stout processes descending into the inner plexiform layer from a cell body in the innermost cell row of the inner nuclear layer (Figs. 7a,b). Output synapses were frequent from labeled processes onto unlabeled cell bodies in the innermost cell row of the inner nuclear layer (Fig. 6), whereas output synapses to the intervaricose segments of unlabeled amacrine cell processes were rather uncommon. The thin postsynaptic processes could not always be positively identified in short series of sections. In no instance, however, did the postsynaptic elements have the characteristics of bipolar terminals, that is, synaptic ribbons or many evenly distributed small synaptic vesicles in an electron dense cytoplasm, or the characteristics of ganglion cells, that is, absence of synaptic vesicles and prominent clusters of ribosomes (Dowling and Boycott, 1966).

The number of input synapses observed onto labeled processes was smaller than the number of output synapses. All of the input synapses were from unlabeled amacrine cell processes; most of them

from amacrine cell varicosities (Figs. 8a,b). However, a few suspected synapses from amacrine cell intervaricose segments to labeled processes were also seen. The input synapses were seen onto both labeled intervaricose segments and varicosities, usually less than 0.6 μ m. The identification of the postsynaptic labeled intervaricose segments was usually difficult, since series of sections had to be analyzed in order to ensure that the intervaricose segment in question was consistently associated with silver grains (Figs. 9a,b,c,d).

Serial synapses were rather commonly seen both in single sections and short series of sections. They included series of up to four amacrine cell processes, where the labeled process could be any one of the postsynaptic elements. One serial synapse was found where the first element was a labeled process, the second was an amacrine cell varicosity and the third element was a bipolar terminal. Several convergent types of synapses were observed with both two amacrine cell processes making synaptic contact with a labeled process as well as two labeled processes contacting an amacrine cell varicosity. A rather complex series of synapses observed is shown schematically in Fig. 10.

DISCUSSION

There is a close similarity between the distribution of radioactively labeled cell bodies and processes in the experimental retinae in this study, both in the light microscope and the electron microscope, and the distribution of dopaminergic cell bodies and processes observed with the Falck and Hillarp technique in earlier studies (Häggendal and Malmfors, 1965; Ehinger, 1966; Dowling and Ehinger, 1978a). Thus, the (3 H)-dopamine-accumulating neurons are identical with the previously described dopaminergic neurons. This conclusion is corroborated by the finding that the distribution of (3 H)-dopamine labeled cell bodies and processes is the same whether the indoleamine-accumulating processes have been destroyed by 5,7-dihydroxytryptamine treatment prior to the (3 H)-

dopamine injection or not. The indoleamine-accumulating neurons are the only other retinal cell population which could be suspected of accumulating (^3H)-dopamine. They appear not to do so to any significant extent in this investigation, most likely because they have a low affinity transport system for dopamine (Florén, 1978). Therefore, they do not accumulate enough (^3H)-dopamine to be visualized with the autoradiographic method. Furthermore, the monoamine oxidase inhibitor Pargyline decreases the turnover of (^3H)-dopamine, which has the effect of increasing the difference between the detectable amounts of (^3H)-dopamine accumulated in the dopaminergic and indoleamine-accumulating neurons.

In the brain, it has proven difficult to use in vivo autoradiographic techniques to label the dopaminergic neurons. Some of the reasons for this are the blood brain barrier and the insufficient diffusion of (^3H)-dopamine in brain tissue, but technical difficulties have also been cited (Nguyen-Legros, Berger and Alvarez, 1981). In contrast, the distinct radioactive labeling of dopaminergic cell bodies and processes and the very low background activity observed after treatment with a monoamine oxidase inhibitor and a single intravitreal injection of (^3H)-dopamine shows, that this simple in vivo technique is indeed quite useful in the study of dopaminergic neurons in the retina, in both the light and electron microscopes. As compared to the 5,6-dihydroxytryptamine labeling method previously used, the autoradiography has the advantage of not requiring the destruction of the indoleamine-accumulating processes nor producing toxic ultrastructural changes in the labeled dopaminergic neurons. On the other hand, it has the disadvantage of a lower resolution, due to the spread of silver grains, which in principle necessitates serial section analysis to ascertain the identification of a labeled process. In addition, the silver grains sometimes occlude important cell structures.

However, as this investigation shows, the unequivocal identification of a labeled dopaminergic process is often achieved even in single autoradiographic sections through the presence of one or several large dense-cored vesicles together with silver grains in the process. Such large dense-cored vesicles were also seen in the dopaminergic processes labeled with 5,6-dihydroxytryptamine, but in those processes the central dense core had often expanded so as to completely fill the vesicles (Dowling and Ehinger, 1978a). Similar large dense-cored vesicles, though often distorted, have also been described in the dopaminergic processes labeled with 5,6-dihydroxytryptamine in the retinae of *Cynomolgus* monkeys and cyprinid fish (Holmgren, 1982; Dowling and Ehinger, 1978b). It is important to note, however, that vesicles larger than 90 nm and with central cores of varying size and density are found in many neurons in the retina, which are not dopaminergic. Some of the large vesicles found in other neurons closely resemble the ones observed in the dopaminergic neurons. For example, distorted large dense-cored vesicles have also been observed in the indoleamine-accumulating neurons, labeled with 5,6-dihydroxytryptamine after the destruction of the dopaminergic processes (Ehinger and Holmgren, 1979; Holmgren-Taylor, 1982b). Thus, the large vesicles with large dense cores cannot be used as a single criterion for identification of the dopaminergic retinal neurons, but they serve as a helpful adjunct to the grain clusters in autoradiography. Similar observations have been made both in the brain and the peripheral nervous system (Hökfelt, 1968; Tranzer and Richards, 1971; for a discussion, see Palay and Chan-Palay, 1975). In this context, it is also noteworthy that the silver grains often appear to aggregate around the large dense-cored vesicles, both in the cytoplasm of the dopaminergic cell bodies and in the dopaminergic processes. It has previously been shown that the large dense-cored vesicles in the peripheral nervous system are able to accumulate neurotransmitter catecholamines (Tranzer and Richards, 1971). The close association between these vesicles and the silver grains observed in this study indicates that this is true in the retina, too.

There is also often a relative absence of silver grains around the presynaptic clusters of small synaptic vesicles in the dopaminergic processes. One interpretation of these observations is that the accumulated (^3H)-dopamine and the endogenous transmitter are transported first to the large dense-cored vesicles and then later incorporated into the small synaptic vesicles. However, the large dense-cored vesicles are not the only storage site in the dopaminergic neurons, because silver grains are found in regions of both cell bodies and processes where no large dense-cored vesicles are present.

Another structural feature of the dopaminergic retinal neurons, revealed with the autoradiographic labeling technique, is the large size of their cell bodies. This large size (mean diameter $9.8 \pm 0.38 \text{ } \mu\text{m}$) as compared to that of other adjacent cell bodies in the innermost "amacrine" cell row of the inner nuclear layer (mean diameter $8.9 \pm 0.27 \text{ } \mu\text{m}$) is evident both in the nucleus and in the wide rim of cytoplasm surrounding it. The dopaminergic neurons in the retina of the cat also belong to the largest amacrine cells (Törk and Stone, 1979).

The (^3H)-dopamine-accumulating neurons in the retina of the rabbit have all the characteristics of amacrine cells. Their cell bodies are situated in the innermost cell row of the inner nuclear layer and their nuclei display typical "amacrine cell" indentations; the processes are confined to the inner plexiform layer and they make conventional synapses like amacrine cells. The processes consist of thin intervaricose segments with mainly microtubules and of rather translucent, small to medium sized varicosities with mainly synaptic vesicles and mitochondria.

The synaptic organization of the (^3H)-dopamine accumulating neurons confirms the earlier findings that they form direct interconnections between amacrine cells only. They have output synapses onto the cell bodies, varicosities and intervaricose segments of

mainly unlabeled amacrine cells. They receive input from other unlabeled amacrine cell processes onto their intervaricose segments and rarely onto their varicosities. The number of input synapses to dopaminergic processes is smaller than the number of output synapses. This has been reported for other animal species also, such as the *Cynomolgus* monkey (Holmgren, 1982). No synapses between two labeled dopaminergic processes have been found in this study, in contrast to the previous study using labeling with 5,6-dihydroxytryptamine. This probably represents only a simple random variation given the relative paucity of such synapses in the previous study.

In the earlier investigation, Dowling and Ehinger also reported that unlabeled amacrine processes made synaptic contact with labeled intervaricose segments only. In this study, a few input synapses have been found from other amacrine processes to small labeled varicosities in addition to synapses to labeled intervaricose segments. This last difference may be explained by the small number of input synapses found in both investigations. Furthermore, the labeling with 5,6-dihydroxytryptamine is to some extent dependent on the presence of cell organelles that can show signs of labeling. There is also a variability inherent in the labeling method with 5,6-dihydroxytryptamine, in that a process may in one section show signs of labeling and in the adjacent section have an almost normal ultrastructure. This explains why some small varicosities which are observed after the accumulation of (^3H)-dopamine may not have been seen after labeling with 5,6-dihydroxytryptamine.

A number of pharmacologically separate subpopulations of amacrine cells have been described in the retina of the rabbit: cholinergic, dopaminergic, GABA-accumulating, glycine-accumulating and indoleamine-accumulating (for review, see Ehinger, 1982). The synaptic organization has been studied in the dopaminergic and the indoleamine-accumulating ones. The two subpopulations differ in

that the dopaminergic ones form interamacrine links, whereas the indoleamine-accumulating neurons mainly have reciprocal synapses with presumably rod bipolar terminals in the innermost part of the inner plexiform layer (Dowling and Ehinger, 1978a; Ehinger and Holmgren, 1979). In other mammals, GABA- and glycine-accumulating neurons are known to contact bipolar cells and ganglion cells in addition to amacrine cells (Pourcho, 1980, 1981; Vaughn, Fami-glietti, Barber, Saito, Roberts and Ribak, 1981).

The observations in this study and a previous one by Dowling and Ehinger (1978a) show that the dopaminergic neurons in the retina of the rabbit form a population of large amacrine cells, which exclusively contact other amacrine cells by means of synapses of the conventional type. Thus, they are interamacrine neurons. Only dopaminergic neurons are so far known to have this synaptic organization. The findings of this study, using auto-radiography and labeling with (^3H)-dopamine after treatment with Pargyline, and the results of the previous work, using labeling with 5,6-dihydroxytryptamine after 5,7-dihydroxytryptamine treatment, are very similar. This close correlation between the two labeling methods shows that they are interchangeable and can be chosen for an investigation according to the requirements of that particular experiment.

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Table I

Distribution of 8109 silver grains in the inner plexiform layer of (^3H)-dopamine injected retinae of rabbits

	Sublaminae of the inner plexiform layer				
	1/5	2/5	3/5	4/5	5/5
% of the total number of grains counted $\pm$ s.e.m.	47.3 $\pm$ 2.69	9.0 $\pm$ 1.55	22.3 $\pm$ 2.67	10.5 $\pm$ 1.75	10.9 $\pm$ 1.58

Table II

Synapses made by the (^3H)-dopamine-accumulating neurons in the inner plexiform layer in the retina of the rabbit

	Sublayer of the inner plexiform layer		
	1/3	2/3	3/3
OUTPUT TO:			
Amacrine cell body	11		
Amacrine process	28	3	1
Bipolar	0	0	0
Ganglion	0	0	0
Unidentified	1	1	1
INPUT FROM:			
Amacrine	9	4	3
Bipolar	0	0	0

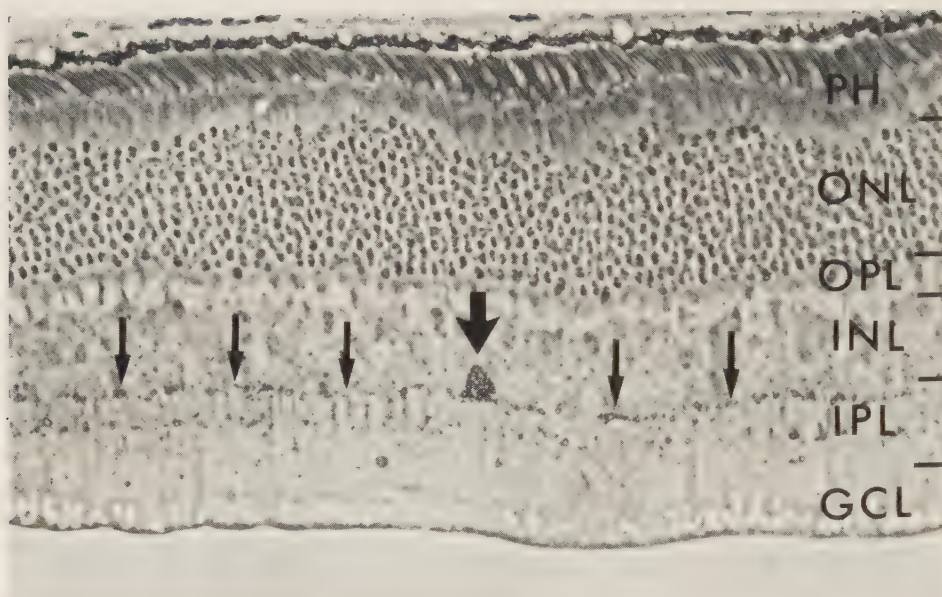


Fig. 1.

Phase contrast micrograph of the retina of a rabbit, injected with 50 μCi (^3H)-dopamine after treatment with Pargyline. The radioactive label is concentrated to a large cell body (thick arrow) in the innermost cell row of the inner nuclear layer and to three bands in the inner plexiform layer. The outermost band close to the inner nuclear layer is the densest one (thin arrows). The middle one is also clearly visible, but the band close to the ganglion cell layer is hardly detectable. Glutaraldehyde- OsO_4 fixation. Ilford L-4 emulsion, exposure 2 weeks, Dektol developer. PH = photoreceptors, ONL = outer nuclear layer, OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer and GCL = ganglion cell layer. X 350.

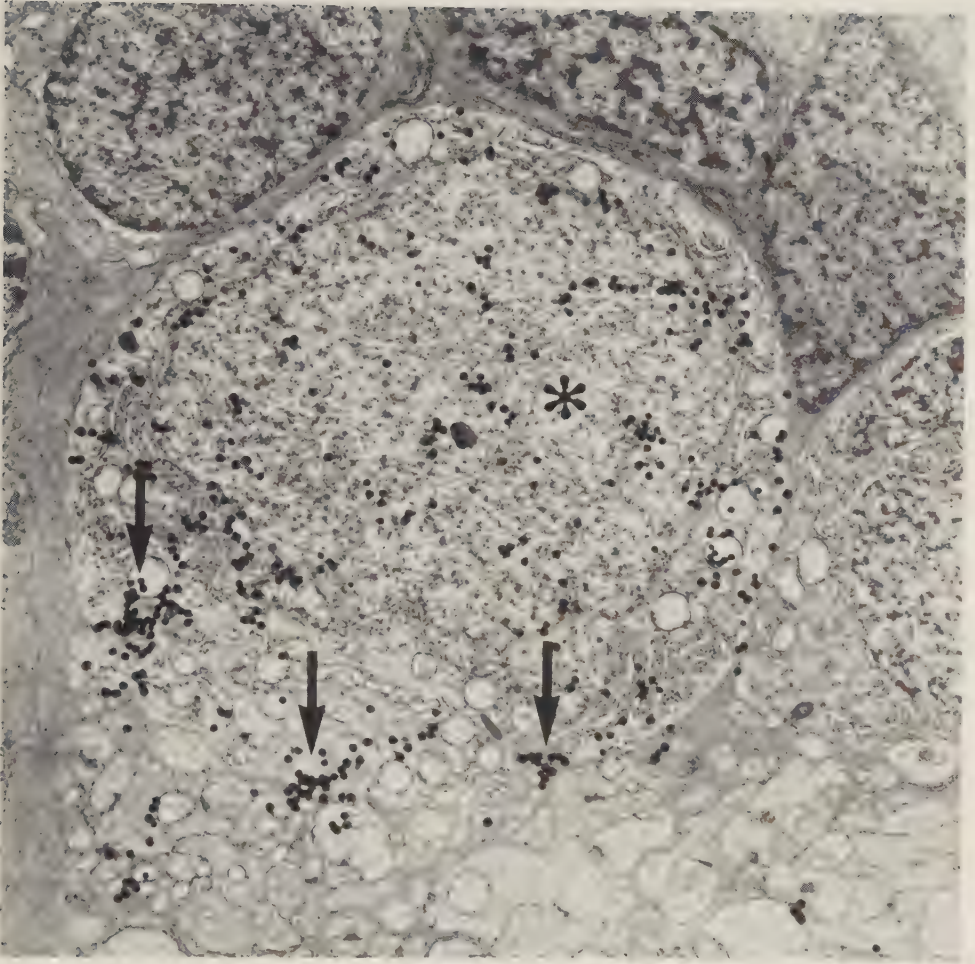


Fig. 2.

Labeled cell body (asterisk) in the innermost row of the inner nuclear layer. The silver grains appear to be randomly distributed in the nucleus, whereas they often aggregate (arrows) around 90-160 nm dense-cored vesicles in the cytoplasm. The labeled cell body is larger and less electron dense than most surrounding cell bodies. Retina treated with Pargyline and (^3H)-dopamine. Glutaraldehyde- OsO_4 fixation, Ilford L-4 emulsion, exposure 11 days, Phenidone developer. X 10 000.

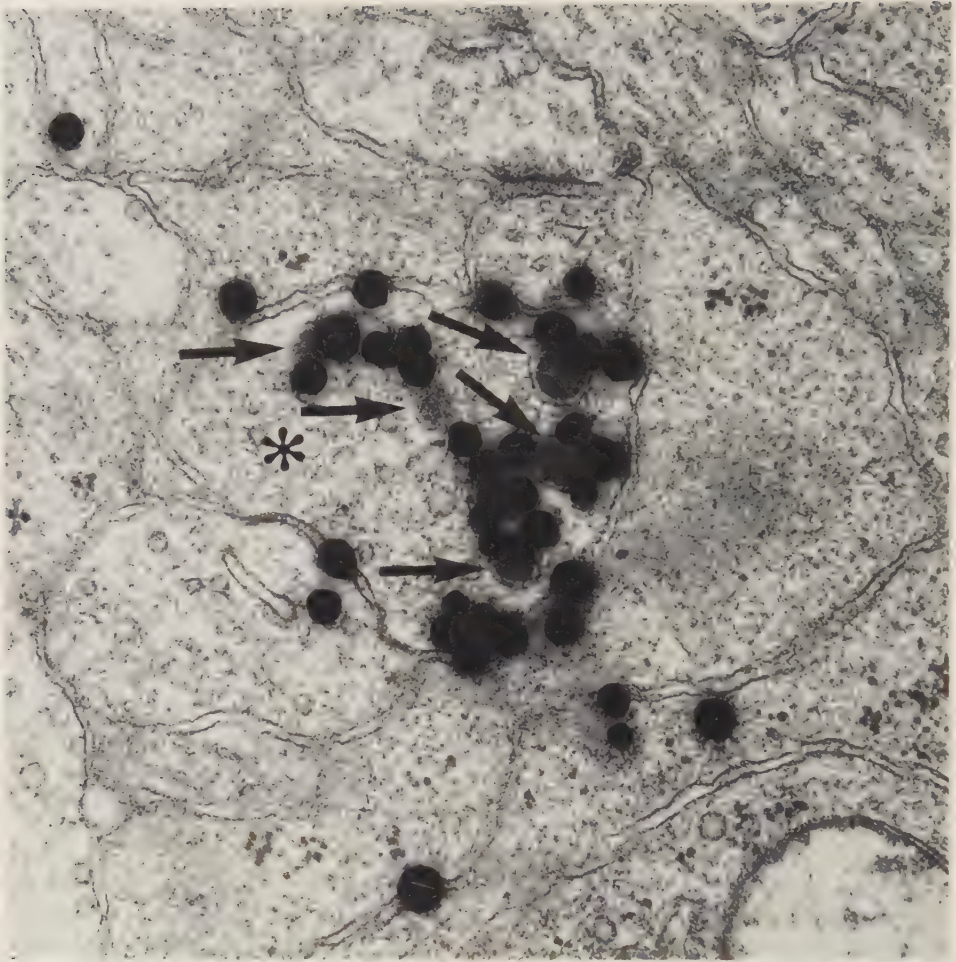


Fig. 3.

Labeled (^3H)-dopamine accumulating varicosity (asterisk) in the outermost sublayer of the inner plexiform layer. The silver grains cluster around large dense-cored vesicles of 90-160 nm diameter (arrows). Between the outer membrane and the dense central core there is a clear space of about 5-12 nm. The rest of the varicosity is filled with small, clear synaptic vesicles of 45-55 nm diameter. Electron micrograph of the retina of a rabbit treated with Pargyline and (^3H)-dopamine. Glutaraldehyde- OsO_4 fixation. Exposure 10 days. X 59 000.

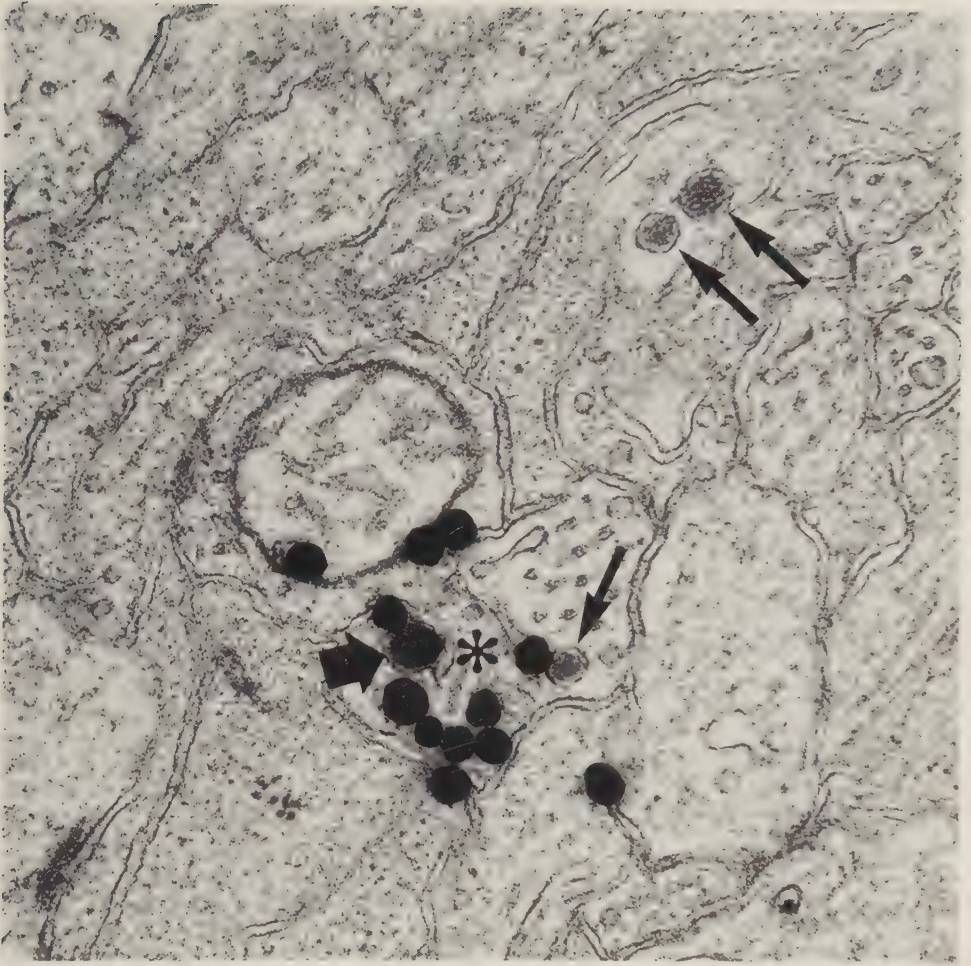


Fig. 4.

Labeled intervaricose segment (asterisk) in the outermost sublayer of the inner plexiform layer. The thick arrow points to a large, 90-160 nm, dense-cored vesicle in the labeled process. The thin arrows point to dense-cored vesicles in adjacent processes. Two of the latter vesicles are clearly smaller in size and all of them have central cores less dense than that of the large dense-cored vesicle in the labeled process. Retina of a rabbit treated with Pargyline and (^3H)-dopamine. Glutaraldehyde- OsO_4 fixation. Exposure 10 days. X 59 000.

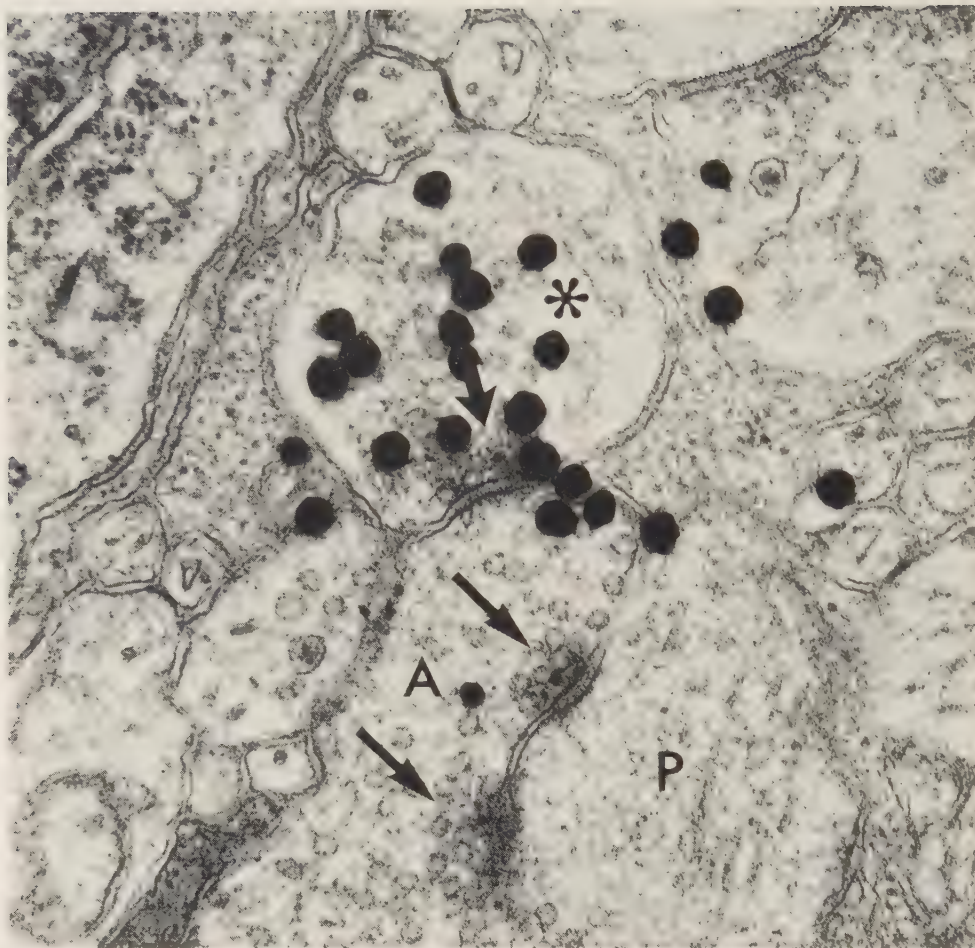


Fig. 5.

Labeled varicosity (asterisk) with output synapse (thick arrow) to an unlabeled amacrine cell process (A) which in turn has two output synapses (thin arrows) to an unidentified process (P). In the labeled varicosity there is a presynaptic cluster of small, clear synaptic vesicles and dense material on the pre- and postsynaptic membranes as well as in the synaptic cleft. Outermost sublayer of the inner plexiform layer. Retina of a rabbit treated with Pargyline and (^3H)-dopamine. Glutaraldehyde- OsO_4 fixation. Exposure 10 days. X 59 000.

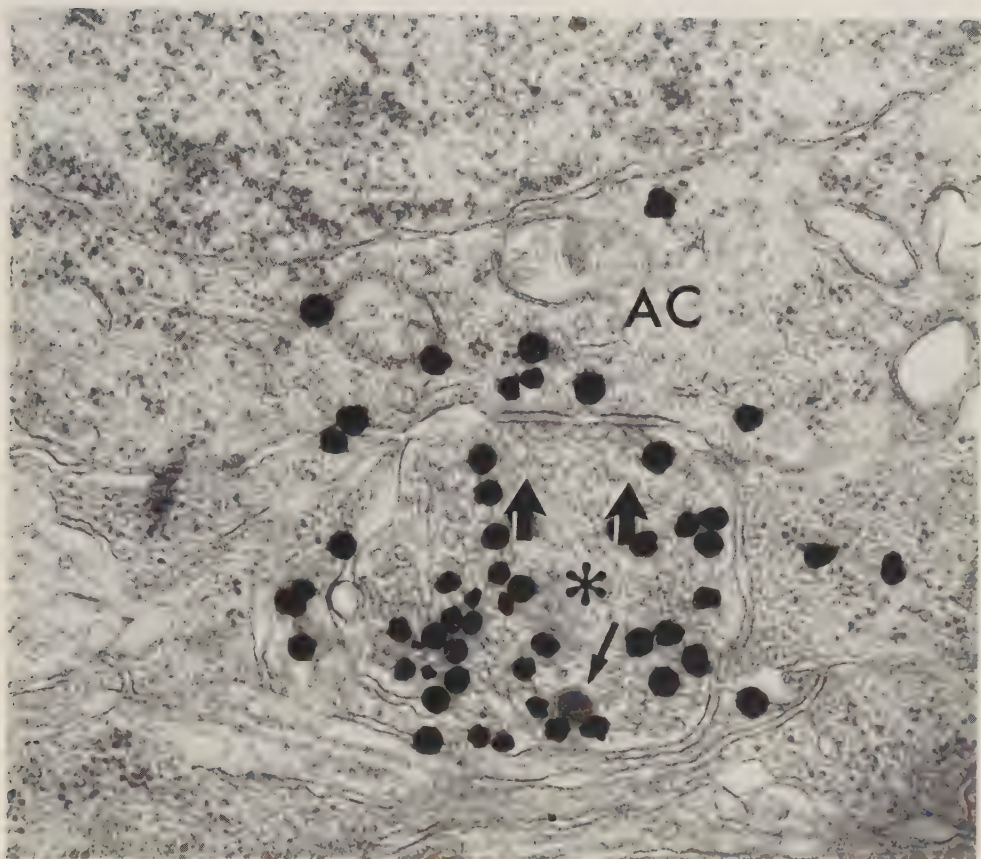


Fig. 6.

Output synapse (thick arrows) from a labeled varicosity (asterisk) to an amacrine cell body (AC) in the innermost cell row of the inner nuclear layer. The thin arrow points to a large, dense-cored vesicle in the labeled process. Retina of a rabbit treated with Pargyline, injected intravitreally with 5,7-dihydroxytryptamine and subsequently with (^3H)-dopamine. Glutaraldehyde- OsO_4 fixation. Exposure 27 days. X 45 000.

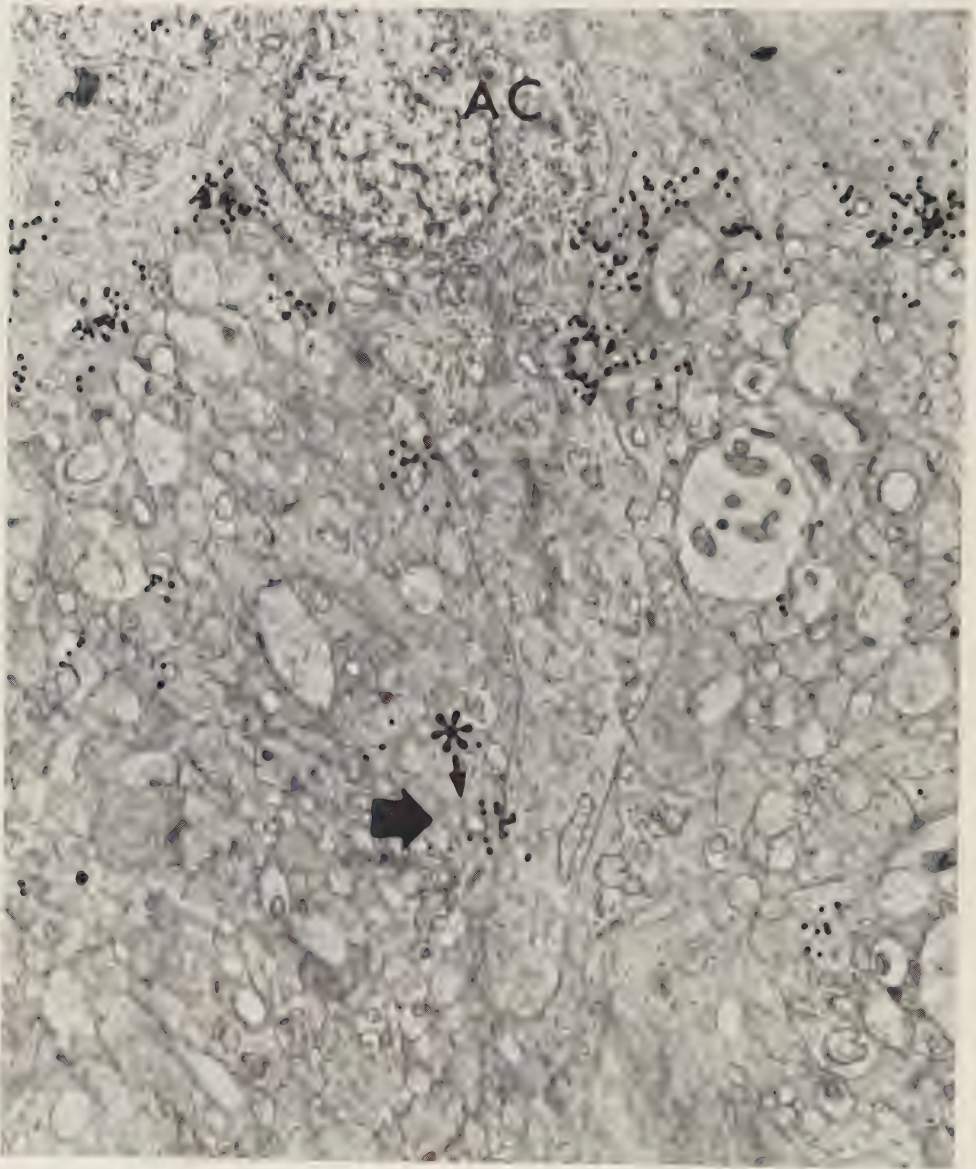


Fig. 7a.

Electron micrograph showing the outermost band of radioactive label in the inner plexiform layer close to the junction of the inner nuclear and inner plexiform layers. Further inwards in the inner plexiform layer, there are aggregations of silver grains corresponding to the middle band of radioactive label. There is an output synapse (thick arrow) from a labeled varicosity (asterisk) to a stout process descending from an amacrine cell body (AC) into the inner plexiform layer. Retina of a rabbit treated with Pargyline and (^3H)-dopamine. Glutaraldehyde- OsO_4 fixation. Exposure 14 days. X 13 500.

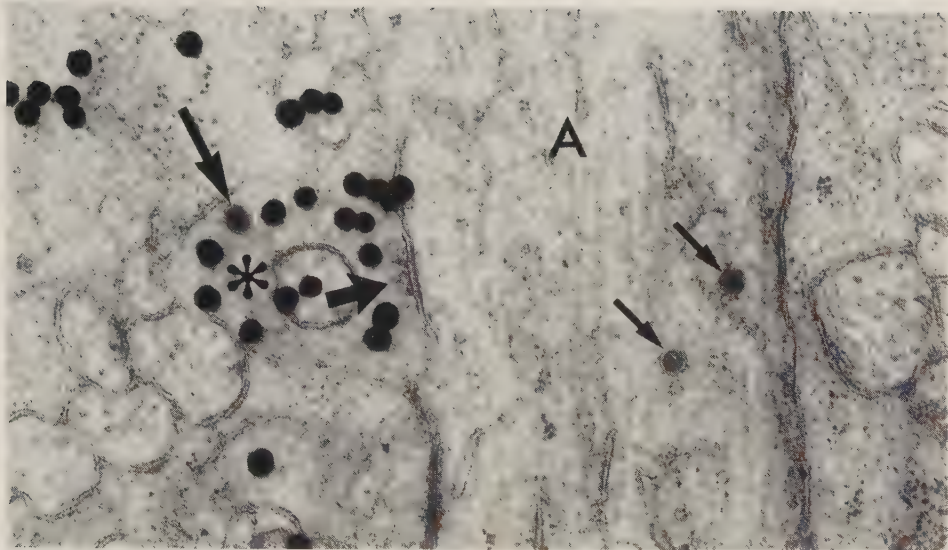


Fig. 7b.

Higher magnification of the output synapse in 7a from an adjacent section. The thick arrow points to the synapse, the long thin arrow points to a large dense-cored vesicle in the labeled process (asterisk) and the short thin arrows point to similar large, dense-cored vesicles in the unlabeled amacrine cell process (A). X 32 000.

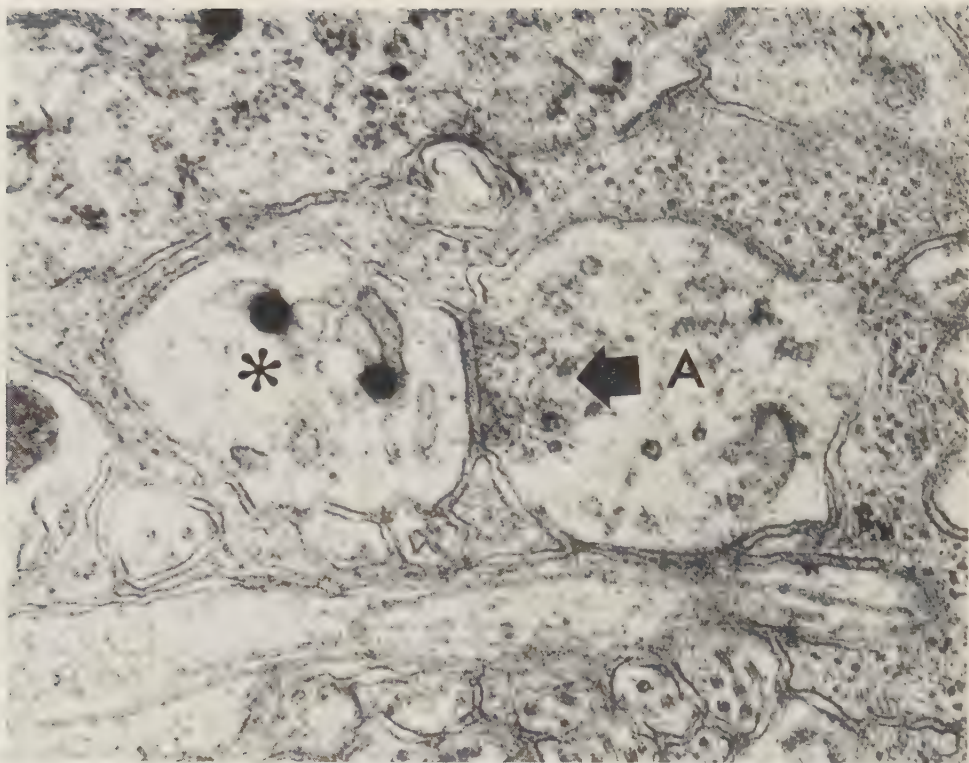


Fig. 8a.

Input synapse (arrow) from an unlabeled amacrine cell process (A) to a labeled small varicosity (asterisk) close to the junction between the inner nuclear and inner plexiform layers. Retina of a rabbit treated with Pargyline and (^3H)-dopamine. Glutaraldehyde- OsO_4 fixation. Exposure 14 days. X 52 500.

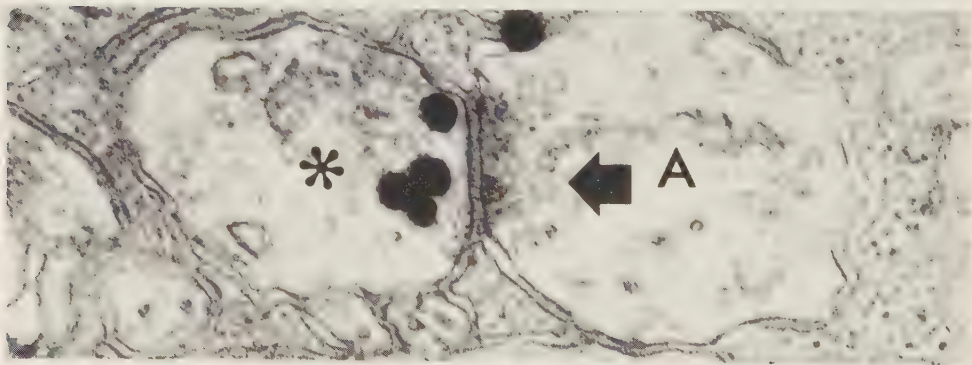
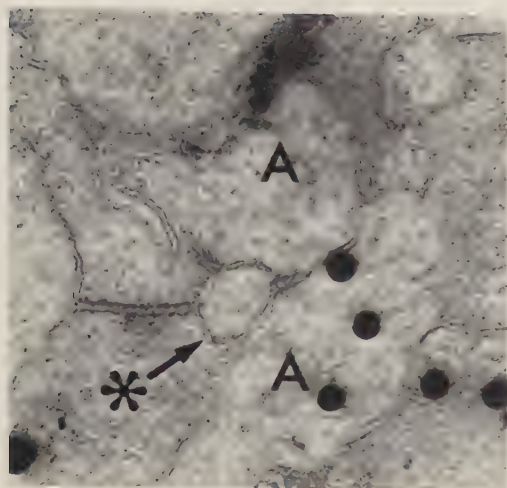
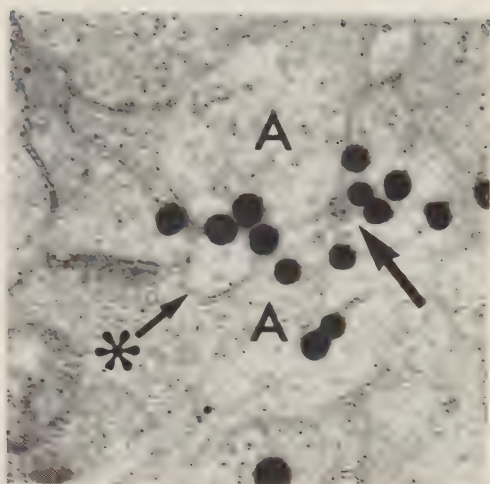


Fig. 8b.

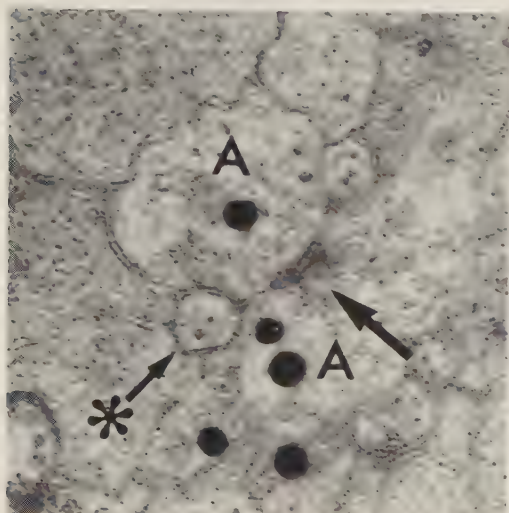
The same input synapse (arrow) as in 8a in an adjacent section. The cluster of silver grains is clearly associated with the labeled small varicosity (asterisk). X 55 000.



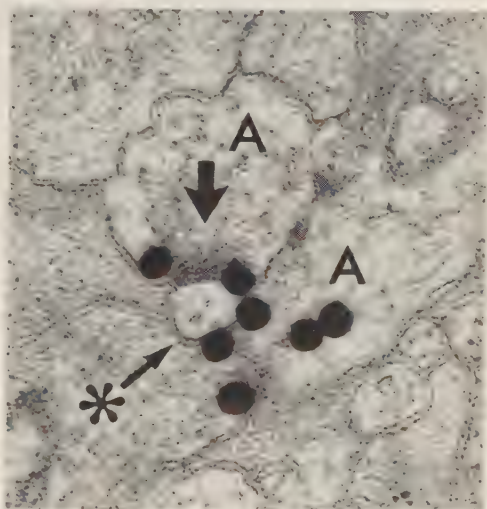
A



B



C



D

Fig. 9.

Serial synapse involving two unlabeled amacrine cell processes (A) and a labeled thin intervaricose segment (asterisk) seen in four adjacent sections. The silver grains cluster around the labeled thin intervaricose segment. a. No synaptic structures are evident. b and c. The large arrow points to an output synapse from one amacrine cell process to the other. d. The second amacrine cell process has an output synapse to the labeled thin intervaricose segment. Outermost sublayer of the inner plexiform layer. Retina of a rabbit treated with Pargyline and (^3H)-dopamine. Glutaraldehyde- OsO_4 fixation. Exposure 13 days. X 34 000.

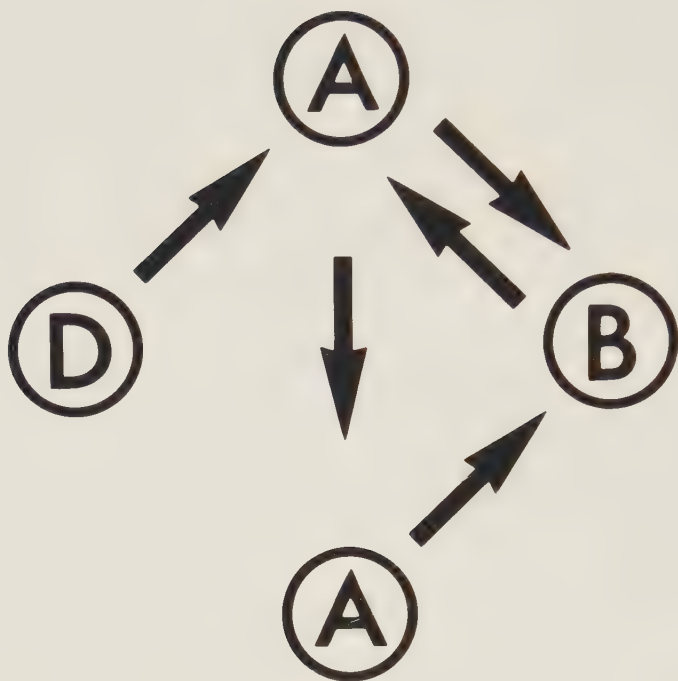


Fig. 10.

Diagram of a complex synaptic arrangement found in the outermost sublayer of the inner plexiform layer. D = (^3H)-dopamine-accumulating process, A = amacrine cell process and B = bipolar cell process.

Electron Microscopy of the Indoleamine-Accumulating Neurons in the Retina of the Rabbit*

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Summary. The recently discovered indoleamine-accumulating retinal neurons were studied electron microscopically after destruction of the dopaminergic retinal neurons and subsequent labeling with 5,6-dihydroxytryptamine. These observations confirm earlier fluorescence microscopical studies on the distribution of the indoleamine-accumulating neurons in the rabbit retina. Their perikarya are known to be located in the inner nuclear layer (INL) among the amacrine cell bodies. Their processes are found only in the inner plexiform layer (IPL), most of them in the innermost third part of that layer. The indoleamine-accumulating terminals are pre- and postsynaptic to bipolar neurons in the innermost sublayer of the IPL. Reciprocal synapses are probably the rule. The synaptic vesicles of indoleamine-accumulating synapses onto bipolar cells are arranged in "globular" clusters around a central electron dense, round body. A number of synapses formed by unlabeled amacrine neurons with postsynaptic indoleamine-accumulating elements were also detected. These synapses were mainly found in the outermost third of the IPL. Synaptic contacts between presynaptic indoleamine-accumulating neurons and postsynaptic unlabeled processes of amacrine cells are very rare.

Key words: Retina – Synapses – Transmitter – Electron microscopy – Rabbit.

A new system of indoleamine-accumulating retinal neurons has recently been described (Ehinger and Florén, 1976, 1978a; Florén, 1979a). During the course of fluorescence microscopical investigation of dopaminergic retinal neurons, it became apparent that intravitreal injection of 5,6-dihydroxytryptamine results in an increase in the number of fluorescent cells, so that not only the dopaminergic neurons but also a second class of retinal neurons show the yellow fluorescence characteristic of 5,6-dihydroxytryptamine. The two populations of neurons can be

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demonstrated simultaneously by injecting α -methyl-noradrenaline together with 5,6-dihydroxytryptamine. The dopaminergic cells then preferentially accumulate the catecholamine and fluoresce green, whereas the indoleamine-accumulating neurons prefer the indole and show a yellow fluorescence (Ehinger and Florén, 1976). The number of indoleamine-accumulating neurons exceeds the dopaminergic ones by a factor of about two, depending on the species studied (Ehinger and Florén, 1976; Florén, 1979a; Dowling et al., 1979). So far indoleamine-accumulating retinal neurons have been detected in the goldfish, rabbit, cat, chicken, pigeon and Cebus monkey, but are absent in Cynomolgus monkeys and humans; and they are not readily demonstrable in rats and guinea pigs (Ehinger and Florén, 1976, 1979; Florén, 1979a; Dowling et al., 1979 and unpublished observations).

Fluorescence-microscopical observations show that the indoleamine-accumulating neurons have their perikarya among the amacrine cell bodies in the inner nuclear layer (INL). Their terminals are exclusively found in the inner plexiform layer (IPL) and mainly in the innermost part in a fairly narrow sublayer (Fig. 1) (Ehinger and Florén, 1976; Florén, 1979a).

For ultrastructural studies some form of labeling of the indoleamine-accumulating neurons is necessary, because no differentiating ultrastructural feature allows their identification. 5,6-dihydroxytryptamine is rapidly accumulated in these neurons (Ehinger and Florén, 1976). In analogy with studies on dopaminergic neurons (Dowling and Ehinger, 1975, 1978a and b), it can be expected that this drug will induce observable structural changes in the indoleamine-accumulating neurons. However, dopaminergic terminals are also labeled by 5,6-dihydroxytryptamine. Methods for chemical destruction of one or the other type of retinal neurons have, however, been devised (Ehinger and Nordenfelt, 1977; Ehinger and Florén, 1978b). For the electron microscopical visualization of the indoleamine-accumulating neurons, the dopaminergic cells can be selectively destroyed by intravitreal injection of 6-hydroxydopamine combined with intraperitoneal injection of the monoamine oxidase inhibitor Pargyline (Ehinger and Nordenfelt, 1977). This method was used in the present study.

By means of this method for identifying the indoleamine-accumulating neurons, it is possible to study their distribution in the retina and their synaptic connections. Knowledge of the morphology of these retinal neurons is a prerequisite for further experiments in order to characterize the neurons electrophysiologically and elucidate their function in the normal retina. Thus, on the basis of morphological and electrophysiological observations, it has been possible to propose that the dopaminergic interplexiform neurons in the goldfish retina regulate the lateral inhibitory effects mediated by horizontal and amacrine cells. Furthermore, it is well known that a great number of drugs, some of which are often used in clinical practice, e.g. in psychiatry, have specific effects both in the retina and the central nervous system (CNS) on the neuronal uptake of biogenic amines including certain indoleamines such as 5-hydroxytryptamine. They are also known to have effects on the retinal receptors. An increased knowledge of the morphology and function of the neurons in the retina and in the CNS will eventually aid in understanding the effects of many drugs on a functional basis, and perhaps even the pathological processes for which the drugs are given.

The indoleamine-accumulating neurons resemble the dopaminergic ones in having a membrane "pump" for certain primary biogenic amines, but as will be shown in this paper, in the rabbit retina they differ from the previously examined dopaminergic neurons (Dowling and Ehinger, 1978a and b) not only in their transmitter and the distribution of their processes but also in the neurons which they contact and in their synapses.

Materials and Methods

Young albino rabbits were injected intraperitoneally with Pargyline (50 mg/kg) and 30 min later with 50 μ g 6-hydroxydopamine intravitreally on two consecutive days. One week later, when the dopaminergic processes had degenerated (Ehinger and Nordenfelt, 1977), an injection of 100 μ g 5,6-dihydroxytryptamine was given intravitreally and after 4 h the eyes were enucleated. Controls were processed in the same way, only the 5,6-dihydroxytryptamine injection was omitted.

Ten experimental and three control eyes were used. Tissue blocks from at least three different parts of the posterior segments were taken.

The posterior segments of all eyes were prepared for electron microscopy in two different ways. Most specimens were fixed in 2% OsO_4 in 0.056 M veronal acetate buffer (pH 7.6–7.8) containing 0.25 M sucrose and 1.8 M CaCl_2 for 1 h in an ice bath and for another half hour at room temperature (West and Dowling, 1972). A few eyes were fixed in ice cold 2.5% glutaraldehyde in 0.065 M cacodylate buffer (pH 7.6) with 4.5 mM CaCl_2 . After a brief wash, the tissue was treated with 2% OsO_4 as described above. In both cases the tissue was dehydrated and embedded in Epon 812, then sectioned and stained with uranyl acetate and lead citrate according to conventional methods and analyzed in a Philips 300 electron microscope.

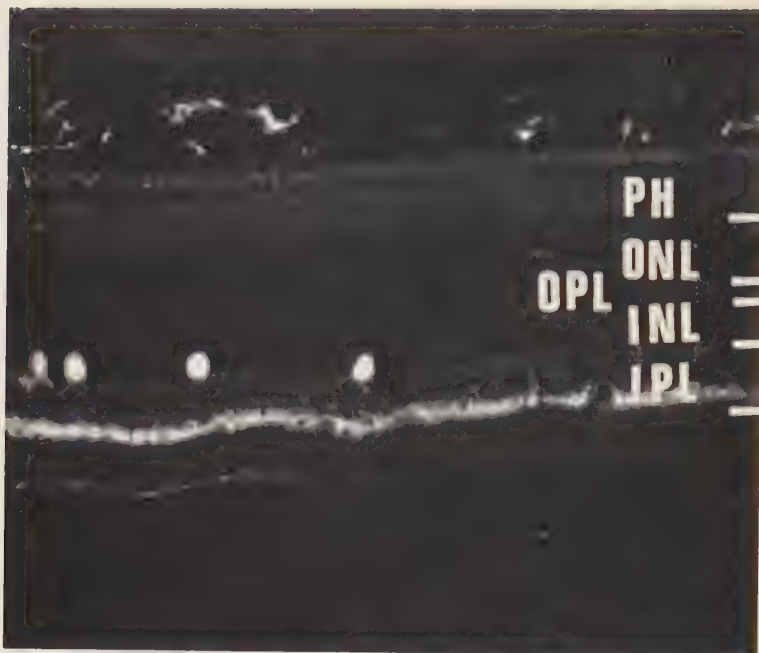
Retinal sections for fluorescence microscopy were prepared by the method of Falck and Hillarp (for a detailed description, see Björklund et al., 1972). In a few experiments the indoleamine-accumulating neurons were destroyed by 5,7-dihydroxytryptamine according to a previously published method (Ehinger and Florén, 1978b).

6-hydroxydopamine HBr and 5,6-dihydroxytryptamine creatinine sulphate were obtained from Regis Chemical Co., Morton Grove, Illinois, USA. All weights given refer to the salts.

Results

Appearance of Labeled Neurons

The labeling procedure produces a number of ultrastructural changes which facilitate the identification of the indoleamine-accumulating neuronal processes. All the labeled processes have the characteristics of amacrine cells, i.e. varicosities alternating with thin intervaricose segments and synapses with a presynaptic cluster of synaptic vesicles (Figs. 2, 3). Most cellular elements including the varicosities, are swollen and their membranes show increased electron density. The cytoplasm is often markedly "empty" with scattered flocculent electron dense material. The features described are especially common in the innermost part of the IPL. Mitochondria, microtubules and synaptic vesicles are found in the labeled neuronal processes in addition to a variety of round structures of varying electron density, some of which presumably are lysosomes. Labeled mitochondria and microtubules have an increased electron density, and the mitochondria are often distorted and appear to be degenerating. The intervaricose segments usually contain only microtubules and occasional synaptic vesicles (Fig. 2).



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Fig. 1. Rabbit retina, 4h after intravitreal injection of 25 μ g 5,6-dihydroxytryptamine. The dopaminergic neurons have been destroyed by the method described in the text. The fluorescent indoleamine-accumulating neurons remain. The perikarya are found in the inner parts of the INL. The neuronal processes are abundant in the innermost third of the IPL, few in the outermost third and rare in the central third of the IPL. *PH* photoreceptors; *ONL* outer nuclear layer; *OPL* outer plexiform layer; *INL* inner nuclear layer; *IPL* inner plexiform layer. Fluorescence micrograph. $\times 275$

Fig. 2. Two labeled processes in the inner sublayer of the IPL. One swollen varicosity is seen with a connecting intervaricose segment. In the intervaricose segment there are labeled microtubules. Both varicosities contain distorted mitochondria (*M*) with increased electron density of their membranes. Synaptic vesicles are also scattered in the cytoplasm of both varicosities. In the swollen varicosity on the right, labeled small synaptic vesicles are seen at the arrow. Opposite one another the cell membranes of the two varicosities are thickened with increased electron density. The intercellular gap in this region is narrow. OsO_4 fixation. $\times 30,000$

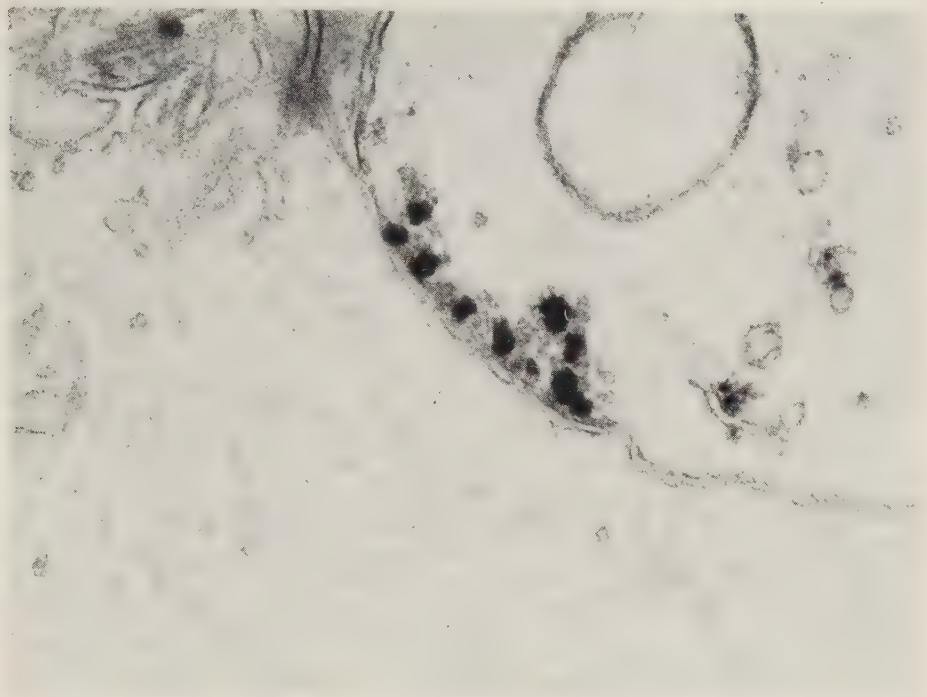


Fig. 3. Synapse from a labeled neuron onto a bipolar cell. Presynaptically, "globular" clusters of synaptic vesicles are present. Small unlabeled synaptic vesicles cluster around larger electron dense structures. OsO_4 fixation. $\times 56,000$

Labeled cell bodies, which are occasionally detected in the INL, show mainly the same ultrastructural changes as the labeled neuronal processes. Membranes and organelles (mitochondria, microtubules and vesicular structures of different sizes) have increased electron density and are often distorted and swollen. The normal cytoplasmic structures are largely replaced by the altered organelles and aggregations of small round, electron dense structures (Fig. 4). These aggregations are irregular in shape and differ in size. They are also present in the nuclear region. The structural changes seen in these labeled cell bodies resemble those found in degenerating perikarya.

The ultrastructural changes most likely represent toxic alterations induced by 5,6-dihydroxytryptamine (Baumgarten and Björklund, 1976; Dowling and Ehinger, 1978a and b). Rarely, membrane structures are seen to surround a completely empty area, at other times the impression of localized glial proliferation is given by the careful "wrapping up" of a labeled process in glial tissue (Fig. 5). These indirect signs of cellular degeneration are the only other degenerative changes seen in the experimental retinas. The degenerating cell bodies are either a result of the labeling procedure or the earlier 6-hydroxydopamine treatment, which is known to cause disappearance of the dopaminergic neuronal processes only, and not of the perikarya (Ehinger and Florén, 1978b).

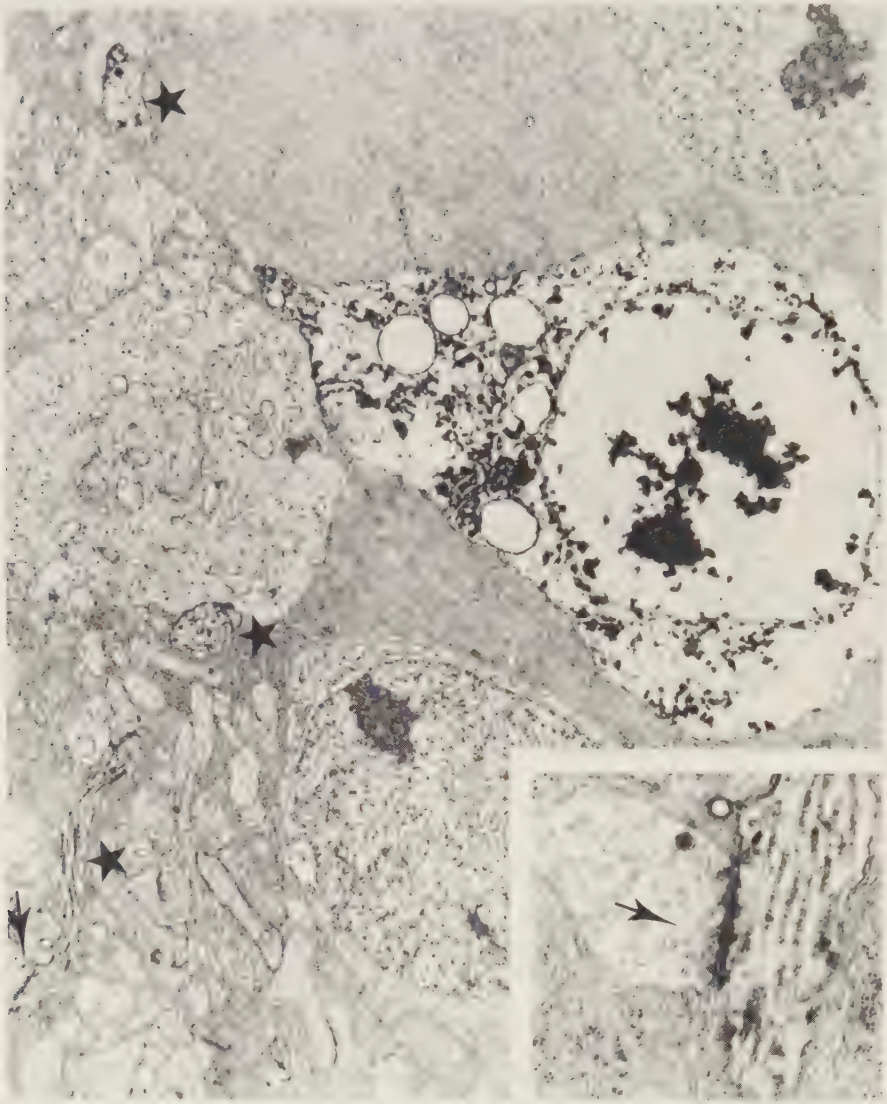


Fig. 4. Labeled cell body in the innermost part of the INL with preterminal part of two processes (stars) which have been followed in serial sections into the outer sublayer of the IPL. There is a synapse from an unlabeled amacrine neuron onto one of the preterminals at the arrow (*inset*). In the cell body there are distorted mitochondria, and aggregations of round electron dense structures are found in the cytoplasm and the nucleus. In the preterminals labeled microtubules are found. OsO₄ fixation. $\times 6400$; *inset* $\times 28,000$

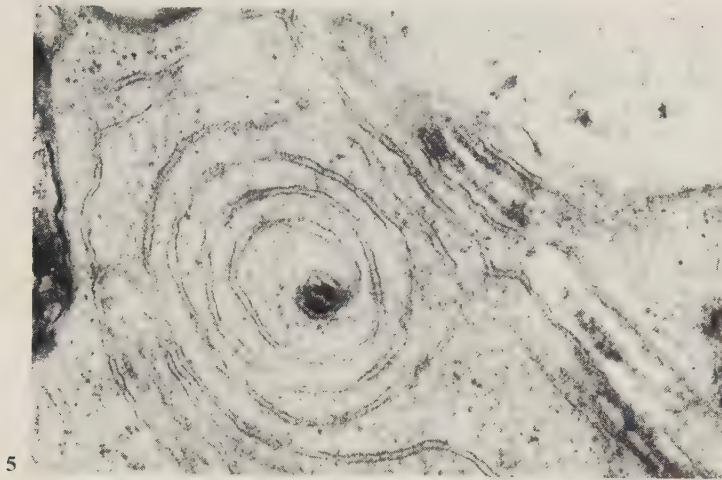


Fig. 5. Labeled narrow intervaricose segment "wrapped up" in a glial process in the inner sublayer of the IPL. OsO_4 fixation. $\times 37,000$

Fig. 6. Synapse from a labeled process onto an amacrine cell, which in turn has a synapse onto another unidentified neuron. The synapse from the labeled varicosity is of the "conventional" amacrine type with a presynaptic cluster of small synaptic vesicles, most of which are unlabeled. Small labeled synaptic vesicles are present in the varicosity at the arrow. Large electron dense round structures, assumed to be lysosomes are seen at star. Inner sublayer of the IPL. OsO_4 fixation. $\times 21,000$

The synaptic vesicles are most often small (40–50 nm in diameter) and round when unlabeled. When labeled they are more pleomorphic and contain an eccentrically or centrally located mass of electron dense material. The characteristic feature in the great majority of outgoing synapses from labeled processes is a presynaptic aggregation of round, electron dense structures having a diameter of approximately 80 to 120 nm (Fig. 3). Empty vesicles of about the same size are often seen in the presynaptic region, but sometimes they are more evenly distributed in the labeled varicosity. Rarely vesicles of the same size possess an electron dense core. Larger vesicles are also found, but infrequently. Occasionally, large vesicles of varying sizes with electron dense membranes and empty cores do, however, completely fill a varicosity. The cytoplasm of such a neuronal process is sometimes considerably darker than the “empty” ones, and more finely granular. Some neuronal processes with very large electron dense, round structures are found. This type of structure is most likely lysosomal in nature and a sign of degeneration (Fig. 6). Electron dense, flocculent material of irregular shape is often found in the labeled processes. Structures resembling multivesiculated bodies have been detected only rarely (Fig. 7).

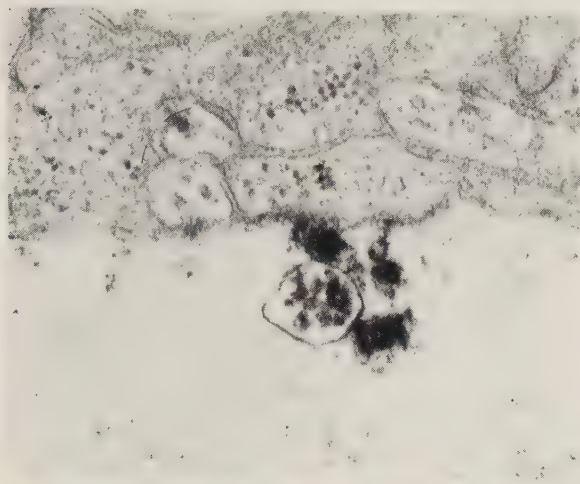
A few labeled processes were found to be continuous with a labeled cell body in the innermost part of the INL (Fig. 4). Labeled neuronal processes are often found juxtaposed to or ensheathed by glial processes. However, this also holds true for the unlabeled processes. To date it has not been proven whether glial processes have a higher affinity to labeled neuronal elements than to unlabeled ones.

Labeled neuronal processes have been found only in the IPL. No labeled processes have been detected in the outer plexiform layer (OPL). A count of 560 labeled processes, noting their location in the IPL was made. The risk of counting the same process more than once was minimized by using sections from different eyes or sections widely apart from one another. The majority of labeled processes (approximately 95 percent) is located in the innermost sublayer of the IPL. Occasional labeled processes are seen in the outermost third of the IPL (approximately 4 percent). The central sublayer of the IPL contains very few labeled processes, not exceeding 1–2 percent. The labeled cell bodies lie among the amacrine cell bodies in the innermost parts of the INL.

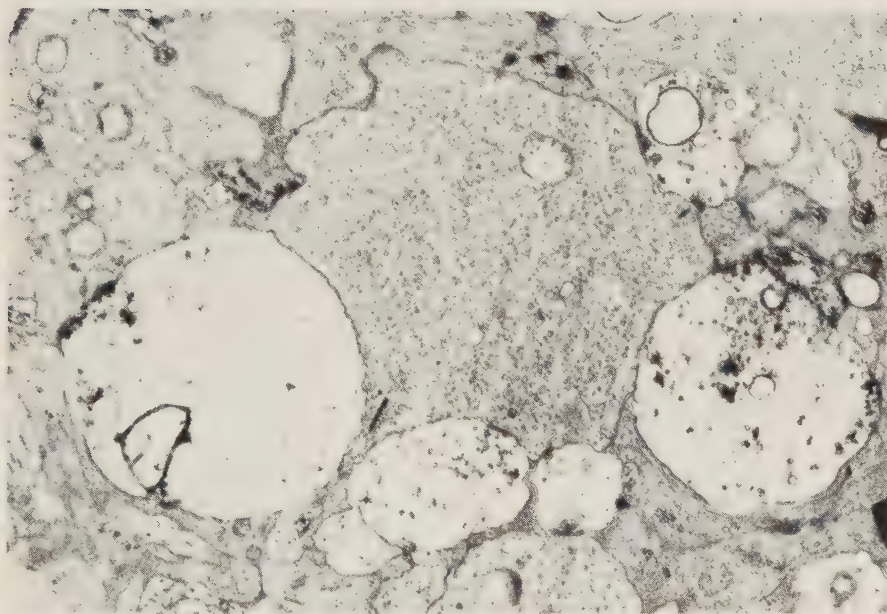
The labeled varicosities in the inner sublayer are often found in groups, usually surrounding a bipolar terminal along with one to three unlabeled amacrine processes (Fig. 8). A substantial number of labeled varicosities, however, lies separated from other labeled processes amidst unlabeled varicosities and intervaricose segments. Single labeled intervaricose segments as well as groups thereof are common in the innermost sublayer. The labeled intervaricose segments are abundant in the regions where the groups of labeled varicosities are most numerous.

The few labeled neuronal processes in the outer sublayers of the IPL usually lie isolated, far apart from one another. They seldom have the swollen appearance of the varicosities just described. Labeled microtubules, small labeled and unlabeled synaptic vesicles as well as vesicular structures of varying sizes predominate.

The appearance and arrangement of labeled indoleamine-acaccumulating processes is consistently the same when labeled processes in different sections from the same eye as well as sections from different experimental eyes are compared. The



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Fig. 7. Multivesicular body surrounded by flocculent electron dense material in a labeled process in the inner sublayer of the IPL. OsO₄ fixation. $\times 48,000$

Fig. 8. Labeled and unlabeled processes of amacrine neurons in a group surrounding a bipolar terminal in the inner sublayer of the IPL. OsO₄ fixation. $\times 13,000$

number of labeled processes does, however, show some variation between different eyes and between different parts of the same eye. The latter phenomenon is probably due to asymmetrical injection of 5,6-dihydroxytryptamine into the vitreous body.

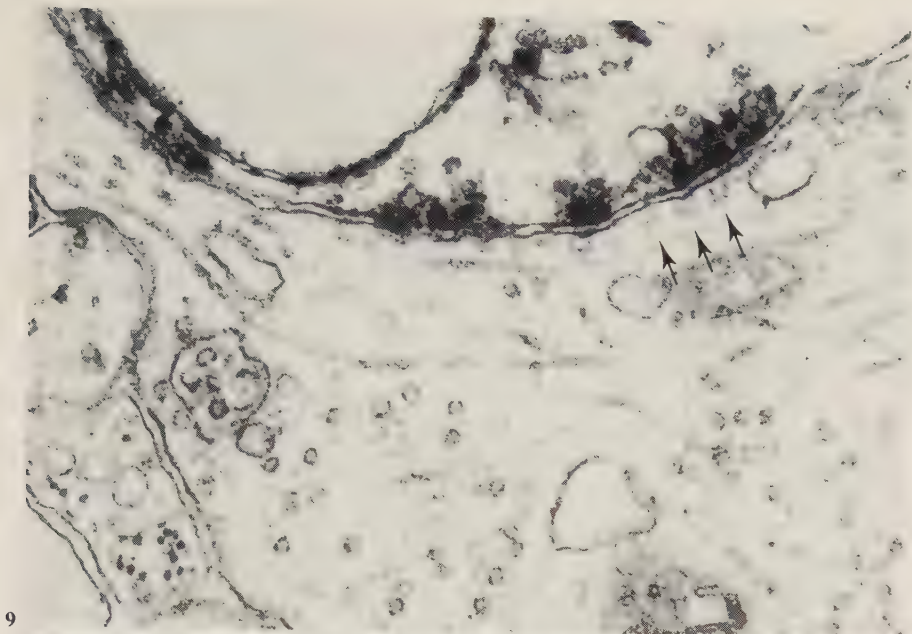
None of the control retinæ treated only with 6-hydroxydopamine one week before fixation showed any of the ultrastructural changes described above.

Synaptic Contacts of the Indoleamine-Accumulating Neurons

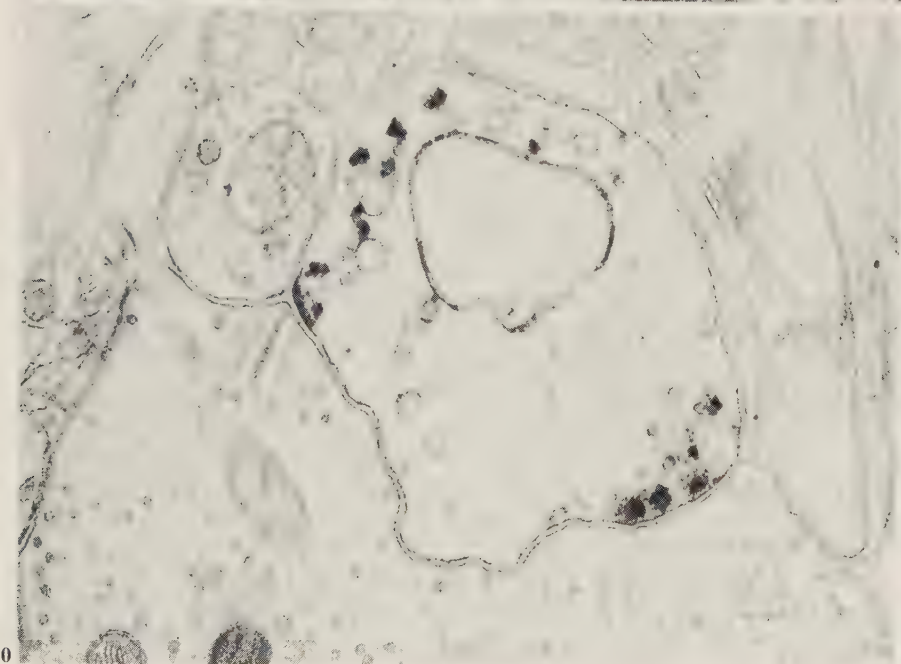
The indoleamine-accumulating neurons form synapses mainly with bipolar neurons. Such synapses are common and thousands of them have been observed. The outgoing synapses of the labeled neuronal processes are only of the amacrine type, i.e. a cluster of synaptic vesicles lies close to the presynaptic membrane. Typically, the round electron dense structures (80–120 nm in diameter) mentioned above are arranged in a row next to the presynaptic membrane, overlying the more numerous smaller unlabeled synaptic vesicles. In fact, the smaller vesicles seem to cluster around the larger electron dense structures forming "globular" clusters of synaptic vesicles (Figs. 3, 9). Occasional empty vesicles of larger size are seen in the presynaptic area, but also scattered in the cytoplasm. The presynaptic membrane sometimes appears slightly thickened and electron dense, but it is often difficult to observe clearly because of the synaptic vesicles. In the synaptic cleft, a poorly visible intercellular material appears to be present. The distance between the pre- and postsynaptic membranes remains constant throughout the synaptic cleft, about 30 to 40 nm. In the bipolar cells contacted by labeled processes no consistent postsynaptic structures or structural changes could be detected. The cell membrane does not show any postsynaptic thickening, but rarely some barely visible irregular fibrillar material is found close to the postsynaptic membrane. Usually, randomly distributed synaptic vesicles are present in the postsynaptic region as in other parts of the terminal. In some instances, a row of synaptic vesicles is seen in close proximity to the postsynaptic membrane. In one bipolar cell, these vesicles had a distinctly "fuzzy" appearance, resembling coated vesicles (Fig. 9).

The synapses from bipolar terminals to labeled indoleamine-accumulating processes are of the bipolar type in the dyad arrangement with synaptic ribbons. The synaptic cleft may be wider than the normal intercellular distance. The postsynaptic membrane of the indoleamine neuron is regularly thickened and has increased electron density (Fig. 10). Often a few vesicles, of synaptic size or slightly larger, cluster close to the postsynaptic membrane thickening. Sometimes these vesicles are empty, sometimes filled with electron dense material.

The types of synapses described above dominate in the inner sublayer of the IPL. Only two synapses have been detected going from indoleamine-accumulating neurons to unlabeled processes, unambiguously identifiable as belonging to an amacrine cell. In addition, a very small number of suspected synapses of this kind have also been found. Most of these synapses are located in the inner sublayer of the IPL. One of these synapses is second in a chain of synapses from a bipolar neuron to a labeled amacrine cell via an unlabeled amacrine cell back to the original bipolar neuron. This synapse (together with two of the suspected ones) seems to have the same presynaptic structures as have been described in the connections with bipolar cells. The other synapses differ in that the presynaptic clusters of vesicles consist only of the small type of vesicles, some of which are labeled and show increased electron density of their limiting membrane or electron dense material located eccentrically or centrally. None of the larger presynaptic electron dense, round structures are seen (Fig. 6). Slightly thickened membrane density occurs pre- and postsynaptically. The synaptic cleft has the same width throughout the synapse and is filled with a barely visible material. In almost all of these synapses minute round



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Fig. 9. Synapse from a labeled process onto a bipolar neuron in the inner sublayer of the IPL. "Globular" clusters of synaptic vesicles are found in the presynaptic region. In the synaptic cleft some electron dense material is barely visible. On the postsynaptic membrane there is a row of "fuzzy" vesicles of synaptic size, resembling coated vesicles (arrows). OsO_4 fixation. $\times 56,000$

Fig. 10. Reciprocal synapse between a labeled process and a bipolar neuron in the innermost third part of the IPL. The synapse from the bipolar neuron onto two amacrine cells, one labeled, the other unlabeled, is in the dyad arrangement. The labeled varicosity shows increased membrane density postsynaptically. A round electron dense structure of approximately the same size as a synaptic vesicle is present in the postsynaptic region. The synapse from the labeled cell process onto the bipolar neuron shows the presynaptic "globular" clusters of synaptic vesicles, but no definite postsynaptic structures are seen. OsO_4 fixation. $\times 36,000$

and semicircular structures are found directly on or juxtaposed to the postsynaptic membrane (Fig. 11). No other specific postsynaptic structures are evident. The labeled processes containing only the small type of synaptic vesicles do not show an increased electron density or thickening of the cellular membranes, but do contain some electron dense, round structures of the largest kind, possibly lysosomes (Fig. 6).

Eighteen synapses from unlabeled to labeled amacrine cells have been detected and again about one third as many synapses are suspected to be of this type. With two exceptions they are all situated in the outermost sublayer of the IPL. One of them represents an ingoing synapse onto a narrow labeled intervaricose segment, the rest of them onto preterminals or middle-sized varicosities (Fig. 12). The labeled neuronal processes possess the characteristics that predominate in the outer part of the IPL. The unlabeled amacrine cell processes have the usual presynaptic cluster of small synaptic vesicles. The presynaptic membrane does not show any specific changes. The synaptic cleft appears to contain the same intercellular substance as has been seen in the other types of synapses and may be slightly widened. The postsynaptic membrane is slightly thickened and has an increased electron density. Aggregations of flocculent electron dense material and small empty vesicles and larger electron dense round structures are located immediately adjacent to the postsynaptic membrane. Both structures resemble the two types of synaptic vesicles described above.

With the exception mentioned above, the in- and outgoing synapses of the indoleamine-accumulating terminals have been found on the varicose parts and preterminals of the neuronal processes. However, often the intervaricose segments of labeled processes are seen to have structures resembling pre- and postsynaptic specializations. No indoleamine-accumulating neuronal process has been observed to have both types of presynaptic structures, i.e. "globular" clusters of synaptic vesicles and conventional "amacrine" clusters of vesicles.

Two juxtaposed labeled processes occasionally show thickening of the cell membranes with increased electron density when the intercellular distance is approximately 20 to 30 nm, i.e. slightly narrower than the usual intercellular distance (Figs. 2, 13). In a small number of cases this phenomenon has been associated with small clusters of more or less irregularly sized vesicles close to the thickened cell membranes. Some of the vesicles are empty, and others electron dense. In only three cases structures resembling synapses were found between labeled processes.

Localized specializations with membrane thickening and clusters of irregular vesicles are also found in labeled neuronal processes abutting glial cells (Fig. 14). In the innermost third of the IPL occasional isolated labeled varicosities show membrane thickening and augmented electron density in a limited region juxtaposing a glial process. A few vesicular structures of sizes corresponding to the two types of synaptic vesicles, some empty and some electron dense, lie near the thickened cell membrane. Some flocculent electron dense material is also present close to the cell membrane. The glial cell does not show any definite specialized structures in such regions.

Neither synapses with ganglion cells nor unambiguously identified outgoing synapses from indoleamine-accumulating neurons to cell bodies of any type have been seen.

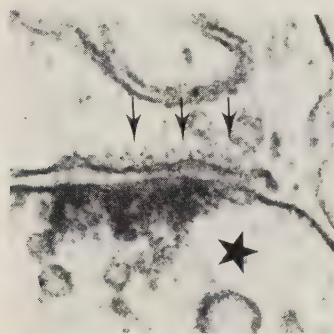


Fig. 11. Synapse from a labeled neuron (star) onto an amacrine cell in the innermost sublayer of the IPL. The presynaptic structures in this synapse resemble the "globular" clusters of synaptic vesicles. There is some electron dense material in the synaptic cleft. On the postsynaptic membrane a row of barely discernible, small semicircular or almost circular structures is found (arrows). OsO₄ fixation. $\times 95,000$

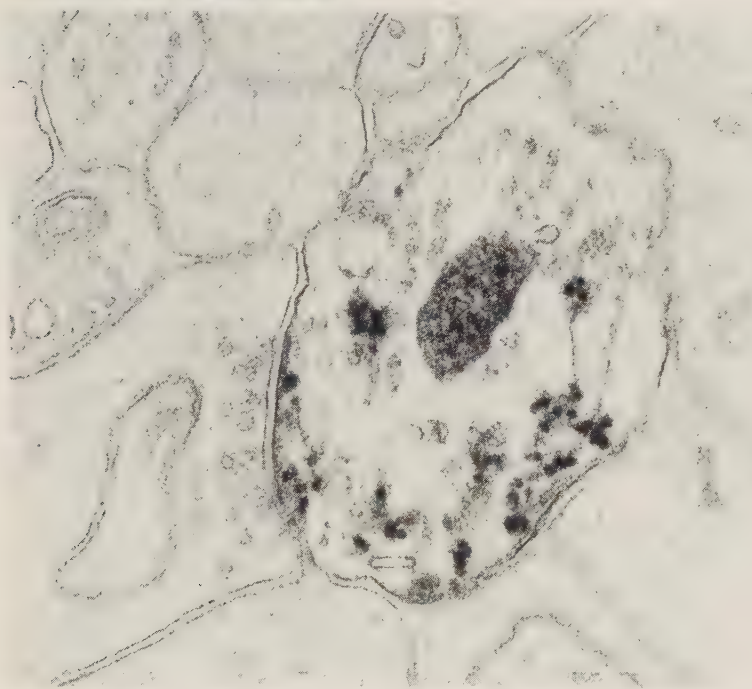
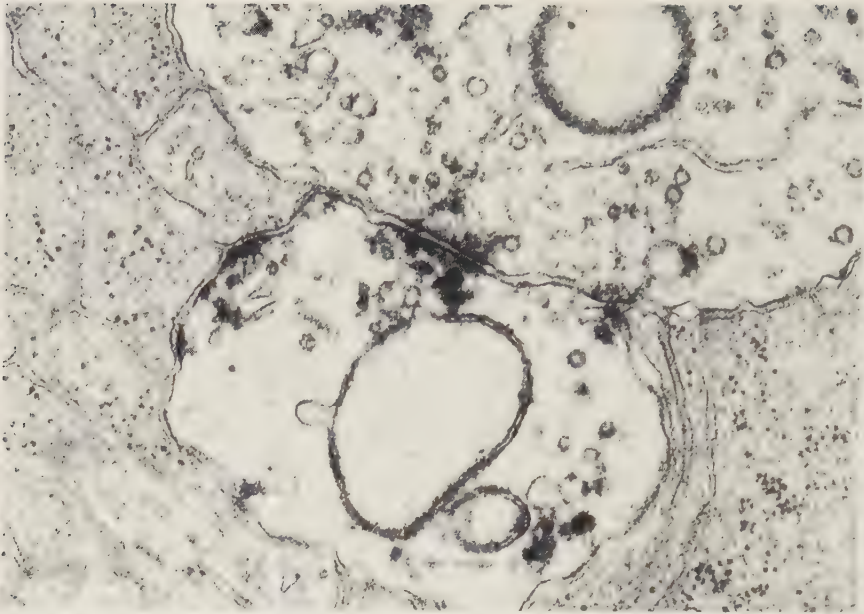
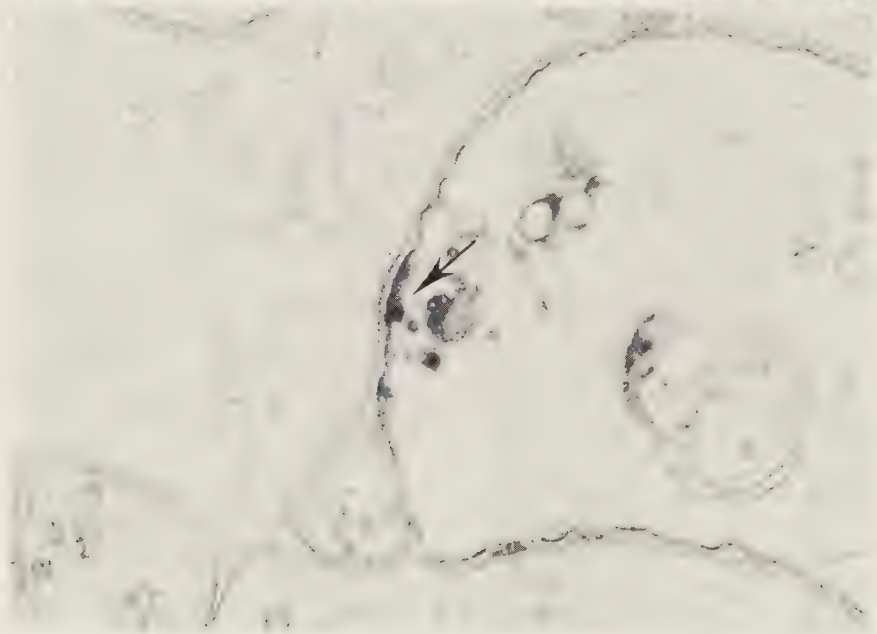


Fig. 12. Synapse from an unlabeled amacrine neuron to a labeled preterminal in the outer sublayer of the IPL. In the presynaptic region there is a conventional cluster of synaptic vesicles. Postsynaptically, there is an increased membrane density and in close proximity to this membrane flocculent and electron dense material. OsO₄ fixation. $\times 56,000$

As noted above, the majority of indoleamine-accumulating terminals and synapses are located in the innermost sublayer of the IPL. Typically a central bipolar varicosity is surrounded by amacrine varicosities; one, two or three of the latter may belong to indoleamine-accumulating neurons (Fig. 8). Some or all of the surrounding amacrine cells, labeled or unlabeled, have synapses onto the bipolar neuron. The bipolar cell in turn possesses one to three outgoing dyad synapses to the surrounding amacrine neurons. If the indoleamine-accumulating neurons are destroyed according to the method described by Ehinger and Florén (1978b), a glial process may take the place of the indoleamine process in the dyad (Fig. 15). So far



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Fig. 13. Two adjacent labeled processes in the inner sublayer of the IPL. In a limited region opposite one another (center of the micrograph) the labeled neurons have increased membrane densities with flocculent electron dense material as well as round, electron dense structures close to the membranes. The intercellular gap is narrow in this region and filled with some electron dense material. Glutaraldehyde and OsO_4 fixation. $\times 56,000$

Fig. 14. Labeled process abutting glial cell in the inner third of the IPL. At the arrow the cell membrane shows an increased density. Two vesicular structures, one with an empty core, the other electron dense, lie close to this membrane thickening. There seems to be some material in the intercellular space, but there is no specific structure in the corresponding region of the glial cell. OsO_4 fixation. $\times 46,000$



Fig. 15. After destruction of indoleamine-accumulating processes by Pargyline and 5,7-dihydroxytryptamine, a bipolar (*B*) dyad is seen to contain one apparently normal amacrine process (*A*). The indoleamine-accumulating process has been replaced by a glial process (*G*). OsO_4 fixation. $\times 36,000$

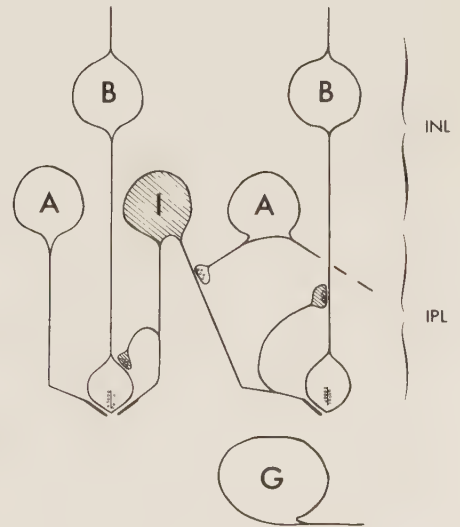


Fig. 16. Synaptic contacts of indoleamine-accumulating neurons in the *IPL* of the rabbit retina. The indoleamine-accumulating neuron (*I*) contacts bipolar cells (*B*) in dyads with reciprocal synapses in the innermost part of the inner plexiform layer (*IPL*). Unlabeled amacrine (*A*) make synapses onto preterminal parts of the indoleamine-accumulating neurons in the outer parts of the *IPL*.

no labeled process has been found in a dyad, where the other process belongs to a ganglion cell. In spite of extensive search, only two bipolar dyads from thousands have been suspected of involving two indoleamine varicosities. Unlabeled amacrine neurons having synaptic contact with a bipolar cell sometimes receive ingoing synapses from other unlabeled amacrine neurons. One such serial synapse seemingly involving two labeled amacrine cells has been detected. The bipolar and the indoleamine-accumulating neurons are very often engaged in reciprocal synapses (Fig. 10). In a few instances a labeled varicosity has been seen to be contacted by two different bipolar neurons.

Apart from the bipolar-amacrine groupings, synapses between single indoleamine-accumulating and bipolar neurons are also found in the inner sublayer. In every section there are also many labeled processes without any synaptic connections. It has not been determined whether labeled varicosities exist without morphologically identifiable synapses. The labeled and unlabeled amacrine cells with synaptic contacts lie separate, surrounded by unlabeled neuronal processes of varying kinds.

Discussion

The labeling procedure employed in the present study produced essentially the same ultrastructural changes in the indoleamine-accumulating neurons as have been described for the dopaminergic retinal neurons by Dowling and Ehinger (1975, 1978a and b). There are, however, some structures and changes which seem to be present exclusively in the indoleamine-accumulating neurons. The typical swollen, empty appearance of labeled varicosities and the presynaptic arrangement with small synaptic vesicles clustering around the larger electron dense, round structures are not seen in dopaminergic neurons labeled in the same way. These structural features dominate in the inner sublayer of the IPL, where the majority of indoleamine-accumulating processes ramify. From the description of the synaptic connections of the indoleamine-accumulating neurons it is clear that the synapses of these neurons, when contacting bipolar cells, are similar to but not identical with the synapses of the dopaminergic retinal neurons. On the other hand, some of the synapses of the indoleamine-accumulating neurons onto amacrine cells lack the "globular" clusters of synaptic vesicles and consequently resemble closely the synapses seen in dopaminergic neurons.

The question can then be raised whether some of the labeled processes found represent surviving processes of dopaminergic neurons. This could explain the difference in presynaptic morphology as well as the difference in appearance between labeled processes, especially when the inner and the outer sublayer of the IPL are compared. Extensive studies with fluorescence microscopy have, however, failed to demonstrate any normal fluorescent dopaminergic processes after treatment with 6-hydroxydopamine according to the method used in this study (Dowling and Ehinger, 1978b; Ehinger and Florén, 1978b; Ehinger and Nordenfelt, 1977). The few remaining ones are swollen and grossly distorted. Considering the extensive disappearance of the dopaminergic processes with the 6-hydroxydopamine method as seen in the fluorescence microscope and the fact that

normal rabbit retina the number of indoleamine-accumulating neurons is known to be at least twice that of the dopaminergic neurons, it seems that the chance of finding a surviving dopaminergic process among the labeled ones is negligible.

Assuming that the chance of encountering a surviving dopaminergic process is small, there are four plausible explanations for the differences in labeled structures and synapses. It is likely that some of the differences in the degree of labeling reflect different states of activity of the neurons. It is known that the amine turnover and the amine uptake increases with increasing neuronal activity (Pletscher, 1975; Andén and Grabowska, 1975; Chan-Palay, 1976). Thus, the more heavily labeled neurons may have been more active than the less intensely labeled cells. The rather clear-cut differences between the inner and outer sublayers are, on the other hand, better explained by the assumption that the ultrastructure of each neuronal process varies with the distance from the cell body or terminal. The third explanation could be the existence of two different kinds of indoleamine-accumulating neurons, possibly with different transmitter substances. This explanation could in particular account for the special appearance of some of the labeled neurons having synapses onto amacrine cells; however, in view of the comparatively few contacts of this kind that have so far been found, the proposal that there exist two kinds of indoleamine-accumulating neurons with bipolar and amacrine contacts, respectively, must remain purely speculative. Increased knowledge as to the identity of the transmitter(s) used by the indoleamine-accumulating neurons may aid in devising labeling techniques which will answer some of the questions that remain unanswered at present. Considering recent speculations that one neuron may indeed use two different transmitters (Burnstock, 1976; Cottrell, 1977), there is today no method of excluding the possibility of one indoleamine-accumulating neuron having synapses onto both amacrine and bipolar cells with different transmitters in different parts of its processes. Finally, if there is in reality only one type of indoleamine-accumulating neuron, the differences in presynaptic morphology would – regardless of the possible existence of one or two types of transmitters in one such neuron – seem to indicate that the presynaptic neuron is in fact able to recognize its postsynaptic partner. Various types of neuronal feedback mechanisms are now supposed to operate in synapses; however, to date insufficient information is available to allow further speculations in this context.

The transmitter of the indoleamine-accumulating neurons has not been identified. These cells do not show any spontaneous fluorescence. From the present study and other experiments (Ehinger and Florén, 1978a), it is clear that the neurons in question possess an uptake mechanism for indoles such as 5,6-dihydroxytryptamine and 5-hydroxytryptamine (serotonin). It is well known that 5-hydroxytryptamine is a transmitter in the CNS (see, e.g. Costa et al., 1974). Initially, 5-hydroxytryptamine was therefore thought to be the transmitter of the indoleamine-accumulating retinal neurons. However, it has not been possible to demonstrate either 5-hydroxytryptamine or 5,6-dihydroxytryptamine in normal rabbit retinas with the histofluorescence method of Falck and Hillarp. Furthermore, the 5-hydroxytryptamine content of the rabbit retina is quite low and the enzyme synthesizing 5-hydroxytryptamine, tryptophan hydroxylase, is absent in the rabbit retina (Florén and Hansson, 1979). Observations on the effect of selective

inhibitors on the uptake of 5-hydroxytryptamine and dopamine in the rabbit retina and on the uptake of 5-hydroxytryptamine in the rabbit retina and CNS also indicate a transmitter similar to but not identical with 5-hydroxytryptamine (Ehinger and Florén, 1978a; Florén, 1979b).

The special structures found in the labeled neurons abutting glial cells are interesting in view of the increasing evidence of glial participation in the neuronal communication system necessary for normal retinal function. It is quite clear that the labeling procedure with 5,6-dihydroxytryptamine produces toxic changes in the neurons accumulating the drug (Baumgarten and Björklund, 1976; Dowling and Ehinger, 1978a and b). Many of the labeled processes are in advanced stages of degeneration. Therefore, the possibility cannot be excluded that a degenerating labeled process can elicit a glial response resulting in the separation of the labeled terminal from its postsynaptic partner by a glial process; hence, the degenerated appearance of the synaptic remnants. Thus, the conclusion cannot be drawn that neurons make contacts of synaptic character with neuroglia.

Attempts to fit the synapses of the indoleamine-accumulating neurons into any of Gray's two types of synapses or the intermediate third category named by others (see e.g. Palay and Chan-Palay, 1976) are only partly successful, because of difficulties in comparing the width of the synaptic cleft to the normal intercellular distance in the photographic material at hand and because of the few synapses onto amacrine neurons so far detected. The synapses onto bipolar neurons with slightly thickened presynaptic membrane density, probably narrow synaptic cleft and no clear postsynaptic membrane density are best placed in the intermediate category. The synapses from bipolar neurons have thicker postsynaptic membrane density than presynaptic membrane density and seem to have a widened synaptic cleft. They may consequently belong to Gray's type 1 synaptic junctions. The synapses from unlabeled amacrine cells appear to have the same characteristics and thus belong to the same type of synaptic junction. The synapses onto unlabeled amacrine cells seem to have equal pre- and postsynaptic membrane densities and may therefore fit into Gray's type 2. The synaptic vesicles in some of these last synapses are also clearly more pleomorphic than the vesicles of the other synapses described. Pleomorphic synaptic vesicles are usually associated with Gray's type 2 or intermediate junctions, and these synaptic junctions have been earlier proposed to be inhibitory in function (Andersen and Eccles, 1965). Type 1 synaptic junctions with round synaptic vesicles would then be excitatory. This concept has, however, been criticized (see Palay and Chan-Palay, 1976). The pleomorphism of the small labeled synaptic vesicles found in this study may be a result of the labeling procedure. In view of this, and the difficulties in classifying the synapses in the study, speculations as to the function of the synapses of the indoleamine-accumulating neurons appear unwarranted at present.

The synaptic arrangement indicates that the main flow of information is between the bipolar neurons and the indoleamine-accumulating neurons in the innermost sublayer of the IPL. The number of reciprocal synapses found between bipolar and indoleamine-accumulating cells also indicates that this is the typical arrangement. The bipolar neurons involved also have contacts with other kinds of amacrine cells. So far, no morphological evidence of synaptic contact between the postsynaptic amacrine cells in the dyad arrangement (when one or two of them are

labeled) has been obtained. After chemical destruction of the indoleamine-accumulating neuron in such a dyad, it is replaced within one week by a glial cell. When observed one week after the removal of its indoleamine partner, the remaining amacrine cell in the dyad is not abnormal in any clearly visible way. This seems to indicate that the two partners in a dyad may function independently from one another.

The number of synapses between the indoleamine-accumulating amacrine cells and other types of amacrine cells are few compared with the bipolar connections. Most of the information from unlabeled amacrine cells to indoleamine-accumulating neurons seems to be given in the outer sublayer of the IPL to the preterminals of these neurons. The outgoing synapses from indoleamine neurons to unlabeled amacrine neurons are too few to allow any general conclusions about this synaptic pathway. Suffice it to say that such synapses do exist and that two types have been seen, one with and one without the presynaptic "globular" clusters of synaptic vesicles. Synaptic connections between two indoleamine-accumulating processes may occur, but are obviously rare.

The general distribution of labeled processes in this study is in accordance with earlier fluorescence-microscopical observations (Ehinger and Florén, 1976). They are much more common in the innermost third of the IPL than in the other parts. This is in contrast to the distribution of the dopaminergic processes, which are most common in the outermost third of the IPL (Dowling and Ehinger, 1978a). However, the indoleamine-accumulating amacrine neurons differ from the dopaminergic neurons not only in their distribution but also in their synaptic connections. The indoleamine-accumulating neurons have synapses mainly with the bipolar neurons, probably generally forming short reciprocal circuits in addition to connecting different bipolar terminals with each other. The dopaminergic amacrine neurons have contacts only with amacrine neurons and not in a reciprocal arrangement. Thus, it seems possible to characterize the amacrine neurons both in terms of their general morphology and synaptic connections, and in terms of their transmitter.

From the results of this study the distribution and the connections of the indoleamine-accumulating neurons may be characterized as shown in Fig. 16. The function of the indoleamine-accumulating amacrine neurons is not known. Like other amacrine cells, they probably act as regulators. With the present knowledge of the synaptic contacts of the indoleamine-accumulating neurons, it may be deduced that they modify the activity of the bipolar neurons by connecting different bipolar cells as well as by feedback mechanisms operating on single bipolar neurons. This modifying activity is also influenced by an input from other non-indoleamine-accumulating amacrine neurons.

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ELECTRON MICROSCOPICAL OBSERVATIONS ON THE INDOLEAMINE-ACCUMULATING NEURONS AND THEIR SYNAPTIC CONNECTIONS IN THE RETINA OF THE CAT

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ABSTRACT

The distribution of indoleamine-accumulating amacrine cells and their synaptic connections in the retina of the cat were analyzed in the fluorescence, phase contrast and electron microscope. The findings were compared to recently characterized morphological subclasses of amacrine cells.

The indoleamine-accumulating neurons were visualized after labeling with an exogenous indoleamine, 5,6-dihydroxytryptamine. The intravitreal injection of the labeling drug was preceded by treatment with the neurotoxic dopamine-analogue, 6-hydroxydopamine, in order to destroy the otherwise interfering dopaminergic processes.

The analysis in the fluorescence and phase contrast microscopes confirmed earlier reports that the indoleamine-accumulating cell bodies and processes have a distribution consistent with that of amacrine cells. A stratified branching pattern of the indoleamine-accumulating processes in the outer half of the inner plexiform layer was discovered. In the inner half of that layer the branching pattern is diffuse.

In the electron microscope the indoleamine-accumulating neurons were seen to have synapses of the conventional type. Their main synaptic contacts are reciprocal synapses with rod bipolar terminals in sublamina b of the inner plexiform layer. They also have synapses with flat cone bipolar terminals in sublamina a, and occasionally with amacrine cells and ganglion cells throughout the inner plexiform layer. Synapses with invaginating cone bipolar terminals, in sublamina b, appear to be rare. The synaptic arrangement with reciprocal synapses with rod bipolar terminals is similar to that of the recently reported AI amacrine cells. It is also similar to that of the indoleamine-accumulating neurons in the retinae of other mammals investigated earlier.

INTRODUCTION

A population of indoleamine-accumulating neurons in the retina of the cat was reported by Ehinger and Florén in '76. They found that when the indoleamine 5,6-dihydroxytryptamine was injected into the vitreous body, a set of neurons, which preferentially took up the indoleamine, was readily demonstrable in the fluorescence microscope with the Falck and Hillarp technique. Similar indoleamine-accumulating neurons have also been demonstrated in other mammals, such as rabbits and Cebus monkeys (Ehinger and Florén, '76; Dowling and Ehinger, '75; Dowling et al., '80); teleost fish such as goldfish (Ehinger and Florén, '76); and cyclostomes and amphibians such as river lampreys and mudpuppies (Ehinger et al., '77; Adolph et al., '80). In all the species investigated the indoleamine-accumulating neurons have a distribution of cell bodies and processes consistent with that of amacrine cells. They are also demonstrable in the electron microscope with the 5,6-dihydroxytryptamine labeling technique, and their synaptic structures and connections have been found to be like those of amacrine cells.

The synaptic organization of the indoleamine-accumulating neurons has been extensively studied mainly in the rabbit and the goldfish, but also in the Cebus monkey and the mudpuppy (Ehinger and Holmgren, '79; Holmgren-Taylor, '82; Dowling et al., '80; Adolph et al., '80). In both of the mammals, the rabbit and the Cebus monkey, the indoleamine-accumulating neurons engage primarily in reciprocal synapses with bipolar terminals which are situated in the border region between the inner plexiform and ganglion cells layers. Such terminals are presumed to be rod bipolar terminals. There is also a small number of synaptic contacts with other amacrine cells.

The synaptic circuitry of the inner plexiform layer in the retina of the cat has also been extensively investigated in recent years. A functional stratification of that layer in terms of ON-center and OFF-center pathways has been demonstrated as well as specific synaptic arrangements for rod and cone channels. Thus, the inner plexiform layer has been divided into two sublaminae, a comprises the outermost third, while b comprises the inner two thirds. In sublamina a, OFF-signals are mediated from flat cone bipolar terminals to the dendrites of OFF-ganglion cells; and in sublamina b, ON-signals are mediated from invaginating cone bipolar terminals to the dendrites of ON-ganglion cells (Famiglietti and Kolb, '76; Nelson et al., '78). Several morphological studies confirm that the processes of the major subclasses of bipolar and ganglion cells ramify and exchange synaptic information in accordance with this lamination (Boycott and Kolb, '73; Kolb and Famiglietti, '74 and '76; Boycott and Wässle, '74; Kolb, '79; Peichl and Wässle, '81; Wässle et al., '81a and b; Kolb et al., '81).

It has further been shown by Kolb ('79) that in the central retina of the cat, flat and invaginating cone bipolar cells have output synapses directly on the dendrites of ganglion cells; whereas rod bipolar terminals have only indirect connections with ganglion cells. All of the rod bipolar synapses in the central retina are with amacrine cell processes (see also Kolb and Famiglietti, '74 and '76; Famiglietti and Kolb, '75). One subclass of amacrine cells, the AII (now A7), has been demonstrated to form a synaptic link between bipolar terminals in sublamina b and the dendrites of ganglion cells in sublamina a (Famiglietti and Kolb, '75; Kolb, '79; Kolb et al., '81). It also connects rod bipolar terminals in sublamina b with flat cone bipolar terminals in sublamina a through chemical synapses, and with invaginating cone bipolar terminals in sublamina b through gap junctions.

In her report of '79, Kolb described another subclass of rod-related amacrine cells, AI, in the central retina, on the basis of serial section analysis in the electron microscope and the study

of Golgi-stained material. The AI was said to have synaptic contacts exclusively with rod bipolar terminals, typically in a reciprocal arrangement and often in conjunction with a process of an AII amacrine cell which formed the other element of the postsynaptic dyad. This synaptic arrangement is similar to that described for the indoleamine-accumulating neurons in the retinae of the rabbit and Cebus monkey previously investigated. This study was initiated, therefore, in an attempt to relate the indoleamine-accumulating neurons to the AI amacrine cells as well as to the ON- and OFF-pathways and rod and cone channels in the retina of the cat.

MATERIALS AND METHODS

Four adult cats, about one year of age, were given intraperitoneally 50 mg/kg body weight of Pargyline, a monoamine oxidase inhibitor, followed half an hour later by an injection intravitreally of 50 ug 6-hydroxydopamine. This procedure was repeated on the next day. As a result, most processes of the dopaminergic amacrine cells were removed (Ehinger and Nordenfelt, '77); this was checked by fluorescence microscopy. If they were present, they would interfere with the electron microscopic analysis, since they also take up the labeling drug, 5,6-dihydroxytryptamine. One week later six eyes were given an intravitreal injection of 50 ug 5,6-dihydroxytryptamine and they were then enucleated after four hours. Two control eyes were only treated with 6-hydroxydopamine one week before enucleation.

Before all intravitreal injections and the enucleations the animals were anesthetized by Nembutal injections intraperitoneally (40 mg/kg body weight) and they were sacrificed by an overdose of Nembutal. Between injections the cats were kept either in the animal room with a daytime illumination of about 100-200 lux and dark at night or in the laboratory with an ambient illumination of about 200 lux.

All intravitreal injections were given with a 50 ul Hamilton precision syringe. 6-hydroxydopamine and 5,6-dihydroxytryptamine were dissolved in 0.9% NaCl with 1 mg/ml ascorbic acid added as antioxidant. After enucleation the eyes were incised at the ora serrata and the anterior segments discarded. Small central and peripheral pieces of each posterior segment were processed for fluorescence microscopy with the Falck and Hillarp method as described by Björklund et al., '72. Retinal sections from those pieces were viewed in the fluorescence microscope with a barrier filter with its 50% cut-off edge at about 500 nm.

The rest of the posterior segments was fixed in 2% OsO₄ in veronal acetate buffer (28 mM barbital sodium and 28 mM sodium acetate) with 66 mM sucrose and 1.8 mM CaCl₂ for an hour in an ice bath and then for another half hour at room temperature. The tissue was thereafter washed, dehydrated in graded alcohols and embedded in Epon 812. For selection of well fixed and well labeled retinal pieces for thin sectioning, sections of 4 um thickness were cut from the Epon embedded material. They were examined in a light microscope at magnifications of 16x and 40x, using phase contrast optics. Thin sections from selected central and peripheral retinal parts were stained with uranyl acetate and lead citrate according to conventional methods. The electron microscope analysis was carried out with a JEOL 100 B and Philips 300 microscope.

Pargyline was obtained from Sigma Chemical Co., St. Louis, U.S.A. 6-hydroxydopamine hydrobromide and 5,6-dihydroxytryptamine creatinine sulfate were obtained from Regis Chemical Co., Morton Grove, Illinois, U.S.A. All weights given refer to the salts.

RESULTS

Light microscopy

The indoleamine-accumulating neurons fluoresce yellow in the fluorescence microscope, with the excitation and emission peaks at about 415 and 520 nm respectively (Björklund et al., '72). Their

cell bodies are found in the innermost two cell rows of the inner nuclear layer, in the ganglion cell layer and the inner plexiform layer; and their processes ramify throughout the inner plexiform layer (Fig. 1). About 83% of 3280 yellow fluorescing cell bodies counted in the retinae of two cats were situated in the inner nuclear layer, about 17% in the ganglion cell layer, and only approximately 0.5% were detected in the inner plexiform layer. The count included both sections from central and peripheral parts of the retinae. The proportions between the numbers of cell bodies found in the three layers were roughly the same from center to far periphery. There were, however, regional differences in the total numbers of cell bodies found in sections from different parts of the retinae. The cell bodies in the central parts were three times more numerous per mm retina (linear distance) than the cell bodies in the far peripheral parts; and the number of cell bodies in the mid-periphery was twice as large as that in the far periphery. The average density of indoleamine-accumulating cell bodies was found to be 20.9 ± 0.99 (s.e.m.) cell bodies per mm whole retina in vertical sections.

Among the strongly yellow fluorescing indoleamine-accumulating cell bodies in the experimental retinae, there are interspersed dopaminergic cell bodies, which are not destroyed by the 6-hydroxydopamine treatment. The dopaminergic cell bodies are recognized because of their greenish yellow fluorescence; in the control retinae and in untreated retinae (Ehinger, 66) their fluorescence is green (emission peak at 470-480 nm, which appears green with the filters used in the fluorescence microscope; maximum excitation at 410 nm, Björklund *et al.*, '72). They are also larger in size than the indoleamine-accumulating cell bodies; they are actually considered to be among the largest amacrine cell bodies in the cat (Törk and Stone, '79). In the inner nuclear layer the dopaminergic cell bodies are situated in the innermost cell row, whereas the indoleamine-accumulating cell bodies are often found in the second cell row. When the experimental retinae

are examined and also compared to the control retinae, where only dopaminergic cell bodies fluoresce, it is seen that the indoleamine-accumulating cell bodies are at least three times more numerous than the dopaminergic ones.

In the inner plexiform layer, there is a dense network of indoleamine-accumulating processes. They form several sublayers in the outer half of the inner plexiform layer (Fig. 1). In most sections, there appear to be three sublayers but sometimes as many as five can be distinguished. In the inner half of the inner plexiform layer, the indoleamine-accumulating processes ramify diffusely with greater density than in the outer half. This branching pattern in the inner plexiform layer is not always evident in the fluorescence microscope, where the ramification of processes sometimes appears diffuse throughout the whole layer. An indoleamine-accumulating process can occasionally be seen to extend into the inner nuclear layer, but they have not been seen to reach the outer plexiform layer. Remaining dopaminergic processes can only very rarely be detected in the inner plexiform layer in the control retinae, and their presence in the experimental retinae is therefore disregarded.

The branching pattern of the indoleamine-accumulating processes can also be studied in detail by light microscopy in sections of OsO_4 -fixed and Epon-embedded tissue after treatment with 5,6-dihydroxytryptamine using phase contrast optics. Labeled cell bodies and processes appear black in such material (Fig. 2). The processes are clearly beaded, that is, varicosities alternate with intervaricose segments. In vertical sections, the sublayering of labeled processes in the outer half of the inner plexiform layer is particularly evident in the phase contrast microscope, and it was first recognized using this method of observation.

There is a certain variability in the number of labeled neurons found in some experimental retinae. In certain retinae the central parts may exhibit the pattern of labeled cells described

above, whereas some peripheral parts may only have a few labeled processes or none at all. In other retinae, it may be that the central parts have few labeled cells, while the peripheral parts have the typical distribution of labeled cell bodies and processes. This variability is considered a consequence of the technique by which 6-hydroxydopamine and the labeling drug are administered to the eye (see DISCUSSION). Only retinal pieces with the characteristic distribution of cell bodies and processes described above was used for electron microscopic analysis.

Electron Microscopy

1. Appearance of labeled neurons

The labeled indoleamine-accumulating processes are readily identified in the electron microscope because of characteristic ultrastructural changes. The membranes of cell organelles such as mitochondria and microtubules usually have increased electron density. Furthermore, the mitochondria are swollen and often empty with only a few short cristae of increased density. The small synaptic vesicles show increased density and/or contain a small eccentric electron-dense spot. The plasma membrane often has increased density in junctional regions and in patches elsewhere (Fig. 3). Two types of labeled processes are seen. Many labeled elements are tightly packed with organelles such as mitochondria and synaptic vesicles and appear generally denser than surrounding, unlabeled processes (Fig. 3). Other processes are quite empty with only scattered aggregations of synaptic vesicles and other organelles (Fig. 4).

Labeled cell bodies also have increased electron density and their cytoarchitecture is often disorganized. The nucleus contains electron dense patches and the cytoplasm has distorted mitochondria, some dilated endoplasmic reticulum and tightly packed ribosome-like particles which occasionally form small aggregations. Occasionally the cytoplasm is completely empty in a distorted part of a cell body or in a varicosity.

2. Distribution of labeled processes

The distribution of indoleamine-accumulating cell bodies and processes observed in the electron microscope is, for the most part, similar to that seen in the fluorescence and phase contrast microscopes. The ramification of labeled processes in the inner plexiform layer appears diffuse with a somewhat denser clustering of varicosities around the large club-like rod bipolar terminals close to border of the inner plexiform and ganglion cell layers, that is, deep in sublamina b (Figs. 6 and 7). A few labeled processes are seen among the cell bodies in the innermost two rows of the inner nuclear layer. There are, however, also a few labeled processes observed within glial cells throughout the inner plexiform, inner nuclear, and outer plexiform layers, and two labeled processes have been seen surrounded by glial cell processes in the outer plexiform layer. All of these elements, found inside or surrounded by glial cell processes, have a severely degenerated appearance, and thus most of them are likely to be remaining dopaminergic processes.

3. Appearance of junctional regions

The indoleamine-accumulating processes have output synapses of the conventional kind: that is, with a presynaptic cluster of small synaptic vesicles close to the presynaptic membrane, dense material on that membrane and in the synaptic cleft and increased density of the postsynaptic membrane, which also has fibrillar or flocculent material deposited on it (Figs. 4,8 and 10). Amacrine cells make synapses of this conventional type (Dowling and Boycott, '66). The indoleamine-accumulating neurons of the cat thus are likely to be a variety of amacrine cell, since both the distribution of their cell bodies and processes and the structure of their synapses are characteristic of amacrine cells. The indoleamine-accumulating neurons have abundant output synapses to rod bipolar terminals. These synapses are usually "punctate", in

that only inconspicuous clusters of small synaptic vesicles are seen surrounding dense, sometimes almost triangular, depositions on the presynaptic membrane (Figs. 3 and 6). When postsynaptic, the indoleamine-accumulating processes usually have a pronounced increase in electron density of the postsynaptic membrane with a prominent aggregation of dense material on it (Figs. 3,4,5 and 9).

In addition to a few conventional synapses between labeled processes, desmosome-like junctions between two indoleamine-accumulating processes with symmetric depositions of dense material on the junctional membranes and dense fibrillar material in the intercellular space have also been detected. Similar desmosome-like junctions between labeled and unlabeled neuronal processes are occasionally seen. The "postjunctional" neuronal process - usually with amacrine characteristics (Dowling and Boycott, '66) - sometimes has dense material on its cell membrane, but is sometimes hard to detect. No gap junctions involving indoleamine-accumulating cells have been seen.

4. Synaptic organization

The kinds of synaptic contacts made by the indoleamine-accumulating neurons in the retina of the cat have been quantified by examining both random single sections and short series of consecutive sections in the electron microscope. The other synaptic elements have been identified using the criteria of Dowling and Boycott ('66). Bipolar cell terminals contain evenly distributed small synaptic vesicles and have ribbon synapses. Ganglion cell dendrites lack synaptic vesicles, and they often display aggregations of ribosomes. Amacrine cell processes have unevenly distributed small synaptic vesicles and synapses of the conventional type already described. The processes of the interplexiform cells have the same characteristics as the amacrine cell ones in the inner plexiform layer (Kolb and West, '77; Nakamura et al., '80). Consequently they cannot be distinguished from one another with the experimental method used in this study.

Further tentative identification of the different subclasses of bipolar cells that have synaptic connections with the indoleamine-accumulating neurons has been based on the distribution of the bipolar cell terminals and the postsynaptic elements of their synapses (Boycott and Kolb, '73; Kolb and Famiglietti, '74 and '76; Kolb, '79). For the most part, flat cone bipolar terminals end in sublamina a of the inner plexiform layer. Both amacrine cell and ganglion cell dendrites are postsynaptic to the flat cone bipolar terminals. Most invaginating cone bipolar processes terminate in sublamina b and have output synapses onto both amacrine cell and ganglion cell dendrites. Finally, rod bipolar terminals are characteristically found deep in sublamina b, in the border region between the inner plexiform and ganglion cell layers. They are often big club-like terminals and in the central retina they only have output synapses onto amacrine cell processes.

Since the indoleamine-accumulating neurons have synaptic connections primarily with bipolar cells, the inner plexiform layer has been divided into equal thirds, designated sublamina a, b₁ and b₂, in this study to facilitate the analysis and the presentation of the results. Sublamina a, b₁ and b₂ then roughly correspond to the levels in the inner plexiform layer, where the terminals of flat cone bipolar cells, invaginating cone bipolar cells and rod bipolar cells respectively are found.

Table 1 shows the kinds and numbers of synapses made by the indoleamine-accumulating neurons. The majority of synaptic contacts made by labeled processes are reciprocal synapses with rod bipolar terminals in sublamina b₂. These rod bipolar processes extend through sublamina b and end with club-like terminals in sublamina b₂. Such terminals are surrounded by labeled and unlabeled amacrine processes (Figs. 6 and 7) and have been seen only to form synaptic contacts with amacrine processes. The indoleamine-accumulating processes clustering around the rod bipolar terminals form input and output synapses with the bipolar termi-

nals. The input synapses from the rod bipolar terminals are ribbon synapses with postsynaptic dyads consisting of one labeled amacrine process and one unlabeled amacrine process. The unlabeled amacrine processes in the dyads have all been identified because they contain small synaptic vesicles. Only in a small number of cases, where the other postsynaptic element in the dyad consisted of a thin intervaricose segment, has positive identification in random or a few serial sections been impossible. The number of reciprocal synapses found between rod bipolar terminals and indoleamine-accumulating processes is so large as to warrant the conclusion that reciprocal synapses are the rule (Figs. 3,6 and 7). In sublamina b_2 about 75% of the reciprocal synapses between rod bipolar terminals and amacrine cell processes appear to involve indoleamine-accumulating processes.

In sublamina b_1 , the indoleamine-accumulating processes have input and output synapses with the thin preterminal parts of rod bipolar processes. There are also reciprocal synapses with rod bipolar processes in sublamina b_1 . In addition, a few bipolar dyads have been seen in sublamina b_1 , where the postsynaptic elements are one labeled process and one verified or suspected ganglion cell dendrite. Bipolar terminals have also been detected in sublamina b_1 , which have ribbon synapses with amacrine and ganglion cell processes as well as amacrine cells and indoleamine-accumulating processes. A few output synapses from indoleamine-accumulating processes onto bipolar terminals with amacrine/ganglion cell dyads have also been found. One reciprocal synapse between such a bipolar terminal and a labeled process has been detected. The bipolar terminals of these synapses are presumably invaginating cone bipolar terminals, which usually end further outwards than rod bipolar terminals and make ribbon synapses onto amacrine/ganglion cell dyads. Thus, it appears that the indoleamine-accumulating processes are pre- and postsynaptic to a few invaginating cone bipolar terminals in sublamina b_1 . However, it should be noted that the number of such synapses

observed so far is small and that they have all been found in the peripheral retina, where the synaptic contacts of the bipolar cells have not been described in detail. One bipolar output synapse onto a dyad of two labeled processes has been discovered in sublamina b_1 .

In sublamina a, the indoleamine-accumulating processes are pre- and postsynaptic to flat cone bipolar cells (Fig. 5). However, in sublamina a only two reciprocal synapses have been detected.

The indoleamine-accumulating processes are pre- and postsynaptic to other amacrine cell processes throughout the inner plexiform layer. The number of input synapses from other amacrine cells observed so far is largest in sublamina a, and they involve both the varicosities and intervaricose segments of the indoleamine-accumulating processes (Fig. 9). Output synapses from indoleamine-accumulating neurons onto other amacrine cell processes appear to be most numerous in sublamina a and b_2 (Fig. 8). A few verified and suspected synapses between two labeled processes have been seen in sublamina a and b_1 (Fig. 4).

A small number of output synapses from indoleamine-accumulating processes onto ganglion cell dendrites have also been discovered. Most of them are located in sublamina a (Fig. 10). The indoleamine-accumulating neurons also make synaptic contacts with neuronal processes ("unidentified" in Table 1) which could not be positively identified in a few serial sections. In nearly all cases, these processes were assumed to belong either to amacrine or bipolar cells.

A variety of serial synapses are seen. One rather common arrangement is one or two output synapses from indoleamine-accumulating processes onto an amacrine cell process which in turn is one of the postsynaptic elements in a bipolar dyad where the second postsynaptic process is an indoleamine-accumulating process

(Fig. 11). This convergent series of synapses has been found in sublamina b_1 and b_2 .

DISCUSSION

The fluorescence microscopy reveals a subclass of indoleamine-accumulating neurons among the amacrine cells; corroborating the observations of Ehinger and Florén '76. In addition, a stratification of the processes of the indoleamine-accumulating neurons in the outer half of the inner plexiform layer has been discovered. This stratification was first and best seen in the phase contrast microscope.

Thus, the labeling with 5,6-dihydroxytryptamine is observable not only in the fluorescence microscope, but also with phase contrast optics, in tissue fixed for electron microscopy and embedded in plastic. Most likely, the labeled neurons are visible in the phase contrast microscope for the same reasons that they are identifiable in the electron microscope. 5,6-dihydroxytryptamine is taken up by the indoleamine-accumulating neurons as a false transmitter. It is in itself osmiophilic and also causes degenerative changes. The fact that the indoleamine-accumulating neurons are clearly visible in the phase contrast microscope is of value when selecting regions for subsequent ultrastructural analysis.

The indoleamine-accumulating amacrine cells have their cell bodies in the innermost two rows of the inner nuclear layer and their processes extend into the inner plexiform layer and the innermost few cell rows of the inner nuclear layer. The very few processes found in the outer plexiform layer in the electron microscope are surrounded by glial processes and are degenerated, thus it is likely that they represent remnants of processes transported to their outward position by the glial cells. They are presumably remaining dopaminergic processes. There is consequently

no significant evidence in this work of the existence of indoleamine-accumulating interplexiform cells.

The number of indoleamine-accumulating neurons decreases from the center to the periphery in much the same way as other classes of retinal neurons in the cat. The gradient is consistent with the existence of a central, specialized visual streak area of maximal visual acuity. The indoleamine-accumulating neurons form a fairly large population of amacrine cells in the retina of the cat as compared to the dopaminergic neurons in the same animal. The indoleamine-accumulating neurons are, in fact, more numerous than the dopaminergic ones in all species investigated (Ehinger and Florén, '76; Florén, '79; Dowling et al., '80; Adolph et al., '80; Ehinger and Åberg, '81). It is not possible to deduce the exact morphology of a single indoleamine-accumulating amacrine cell from the light microscopy of vertical sections only. Presumably it is a wide-field diffuse cell with beaded processes.

The variability in the number of labeled indoleamine-accumulating neurons within some experimental retinae has been noted before (Ehinger and Holmgren, '79; Holmgren-Taylor, '82). It is assumed that asymmetry of injection of both the neurotoxic 6-hydroxydopamine and the labeling drug, 5,6-dihydroxytryptamine, is responsible at least in part for this variability as well as possibly different functional states of the neurons. As noted before, there is no pattern in the variability.

The ultrastructural changes in the labeled neurons consist of increased density of most membranes in the cell and often also of swelling of different cell components. They are similar to those previously seen (Ehinger and Holmgren, '79; Dowling et al., '80; Adolph et al., '80; Holmgren-Taylor, '82).

Figure 12 summarizes the synaptic connections of the indoleamine-accumulating neurons. They have conventional output synapses and consequently fulfill all criteria for being amacrine cells.

Their main synaptic connections are reciprocal synapses with rod bipolar terminals in sublamina b. Further, there are synapses with flat cone bipolars in sublamina a, and with other amacrine cells throughout the inner plexiform layer. A small number of output synapses onto ganglion cells are present as well as a few inter-connecting synapses between indoleamine-accumulating processes. The synapses with presumed invaginating cone bipolar cells, so far found only in the peripheral retina, are not shown in the diagram.

From this synaptic organization it can be seen that the indoleamine-accumulating neurons are likely to play their most important role in the rod pathway. They probably also form links between the ON- and OFF-pathways through their widespread connections with all classes of retinal neurons in both the ON- and OFF-strata of inner plexiform layer.

In the other mammals (rabbit and Cebus monkey) where the synaptic organization of the indoleamine-accumulating neurons has been studied, the main synaptic connections of these cells have also been reciprocal synapses with bipolar cells (presumably rod bipolar cells) in the innermost part of the inner plexiform layer (Ehinger and Holmgren, '79; Dowling et al., '80). In teleost fish (goldfish and carp), however, the indoleamine-accumulating neurons form mainly interamacrine links although contacts with bipolar cells are also present (Holmgren-Taylor, '82). This species difference in the synaptic organization of neurons with the same putative transmitter is also seen, for example, with the dopaminergic neurons. They are, in the rabbit and Cynomolgus monkey, amacrine cells with interamacrine contacts (Dowling and Ehinger, '78a; Holmgren, '82); whereas they are interplexiform cells with a centrifugal flow of synaptic information in teleost fish and Cebus monkeys (Dowling and Ehinger, '75 and '78b; Dowling et al., '80).

Of the various amacrine cell types described by other investigators on the basis of serial section analysis of both Golgi-stained and untreated retinae, the AI amacrine cell (Kolb, '79)

was described as always engaging in reciprocal synapses with rod bipolar terminals in sublamina b. Since the indoleamine-accumulating cells share this characteristic with the AI cells, they could be identical. However, this study shows that the indoleamine-accumulating neurons also make synaptic contacts with flat and probably invaginating cone bipolar cells, as well as with other amacrine cells and ganglion cells. Furthermore, unlabeled amacrine cells with reciprocal synapses with rod bipolar terminals in sublamina b have also been observed. Thus, it is not likely that all AI cells are indoleamine-accumulating.

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Table 1
TOTAL NUMBER OF SYNAPSES

	IPL		
	a	b ₁	b ₂
<u>Output to:</u>			
Bipolar	15	11	>100
Amacrine	14	5	15
Ganglion	8	2	2
Unidentified	24	4	18
<u>Input From:</u>			
Bipolar	12	10	>100
Amacrine	9	2	2
<u>Reciprocal:</u>			
Bipolar	2	5	> 50
Amacrine	0	0	0
<u>Labeled to Labeled:</u>			
	3	2	0

Note that the output and input synapses of the reciprocal synapses are also included under the OUTPUT and INPUT headings.

Abbreviations

A	amacrine cell	IPL	inner plexiform layer
FB	flat cone bipolar cell	M	mitochondrion
G	ganglion cell	ONL	outer nuclear layer
GCL	ganglion cell layer	P	unidentified neuronal process
IB	invaginating cone bipolar cell	RB	rod bipolar cell
INL	inner nuclear layer		

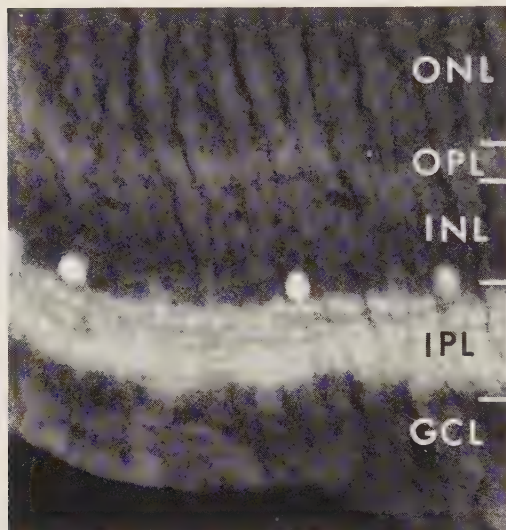


Fig. 1.

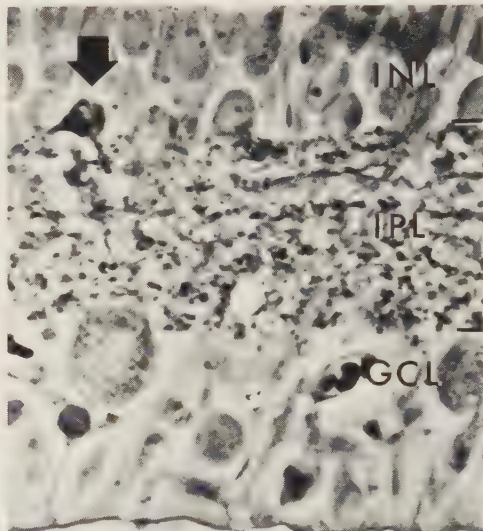


Fig. 2.

Fluorescence micrograph of a cat retina, in which the dopaminergic processes have been removed by treatment with 6-hydroxydopamine and the indoleamine-accumulating processes have been labeled with 5,6-hydroxytryptamine. Several bands of fluorescing indoleamine-accumulating processes are seen in the outer half of the inner plexiform layer, whereas diffusely distributed, fluorescing processes ramify in the inner half. The cell bodies fluoresce strongly yellow and are consequently indoleamine-accumulating. Mid-peripheral retina. X 350.

Fig. 2.

Phase contrast micrograph of an experimental retina which has been fixed in OsO_4 and embedded in Epon 812. A labeled cell body with two processes entering the inner plexiform layer is seen at the arrow. Labeled processes in the inner plexiform layer appear as black dots and lines. Three to four bands of processes are distinguishable in the outer half of the inner plexiform layer, whereas the ramification of processes is diffuse in the inner half. Mid-peripheral retina. X 680.

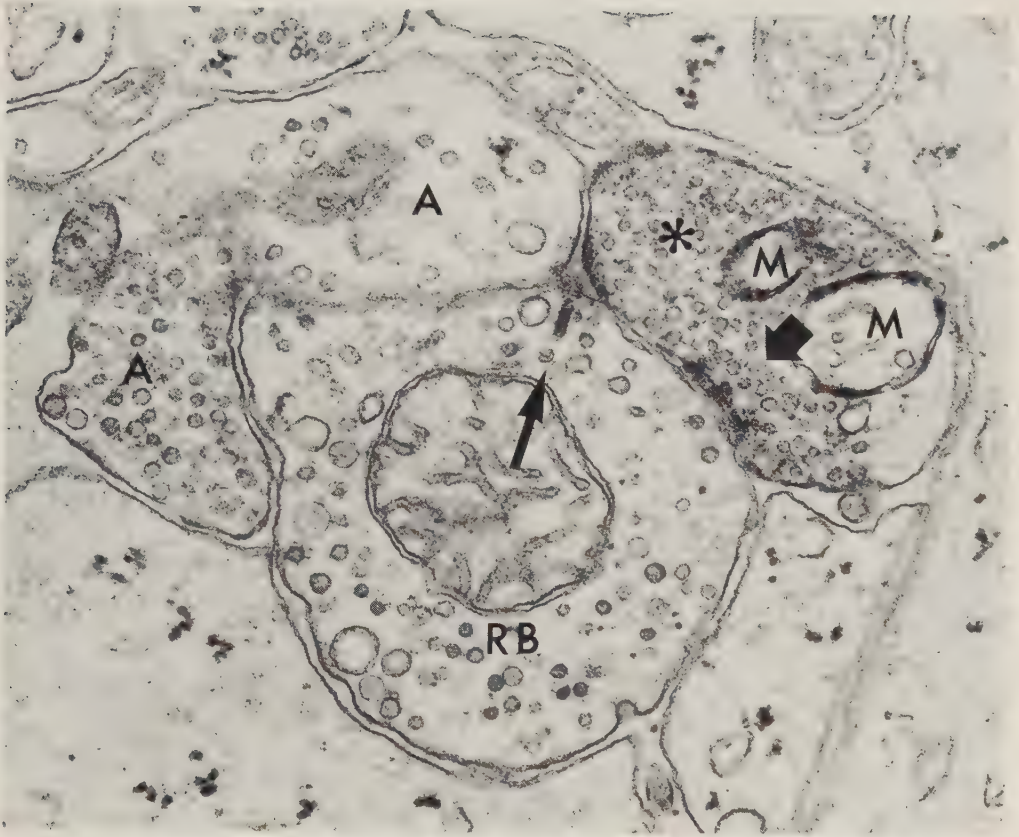


Fig. 3.

Labeled process (asterisk) almost completely filled with small dense synaptic vesicles. Two mitochondria (M) with electron-dense outer membranes are also seen in the process. The labeled process has a reciprocal synapse with a rod bipolar terminal (RB). The output synapse (thick arrow) from the labeled process to the rod bipolar terminal has an inconspicuous cluster of small synaptic vesicles around dense depositions on the presynaptic membrane. There is a synaptic ribbon (thin arrow) in the bipolar terminal and dense material in the synaptic cleft between the bipolar terminal and the two postsynaptic processes. The postsynaptic dyad consists of one labeled and one unlabeled amacrine process (asterisk and A respectively). The dense material on the postsynaptic membrane of the labeled process is particularly evident. Electron micrograph of border region between the inner plexiform and ganglion cell layers, sublamina b₂. Central retina. OsO₄ fixation. X 43,000.

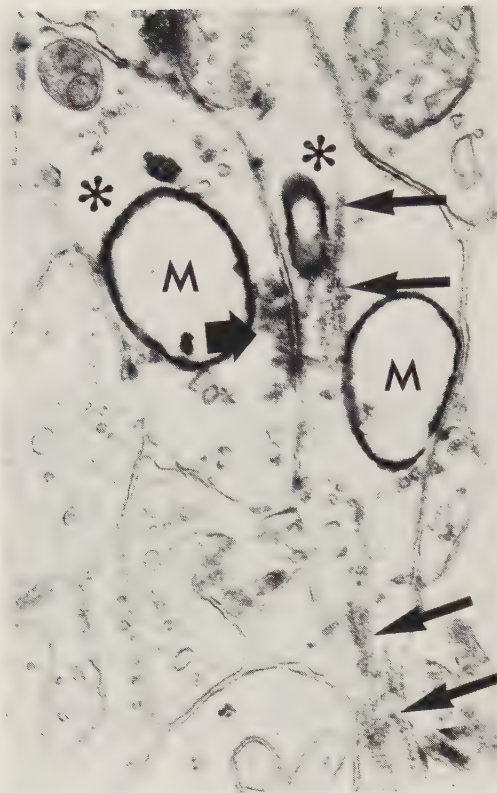


Fig. 4.

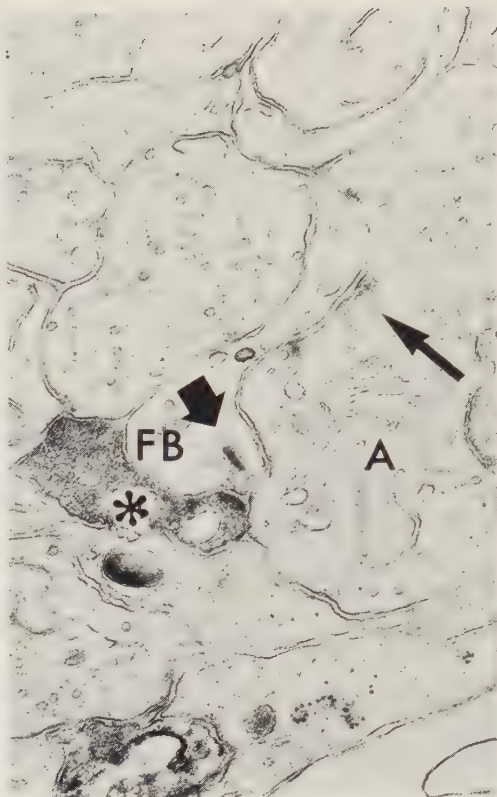


Fig. 5.

Electron micrograph of two adjacent indoleamine-accumulating processes (asterisks) in the outermost third of the inner plexiform layer, sublamina a. Two swollen empty-looking mitochondria with dense outer membranes are marked M. Microtubules of increased electron density are present in one of the varicosities and the adjoining intervaricose segment (thin arrows). There are scattered small synaptic vesicles in the varicosities. One of the labeled varicosities makes synaptic contact (thick arrow) with the other one. There is a pre-synaptic cluster of small synaptic vesicles, and dense material on the pre- and postsynaptic membranes and in the synaptic cleft. Peripheral retina. OsO₄ fixation. X 31,000.

Fig. 5.

Output synapse (with ribbon, thick arrow) from flat cone bipolar terminal (FB) in sublamina a to one labeled and one unlabeled amacrine cell process (asterisk and A respectively). The labeled process is almost completely filled with small synaptic vesicles and has a prominent postsynaptic membrane deposition. The unlabeled amacrine cell process has an output synapse (thin arrow) back to the bipolar terminal, thus participating in a reciprocal synapse. Central retina. OsO₄ fixation. X 25,000.

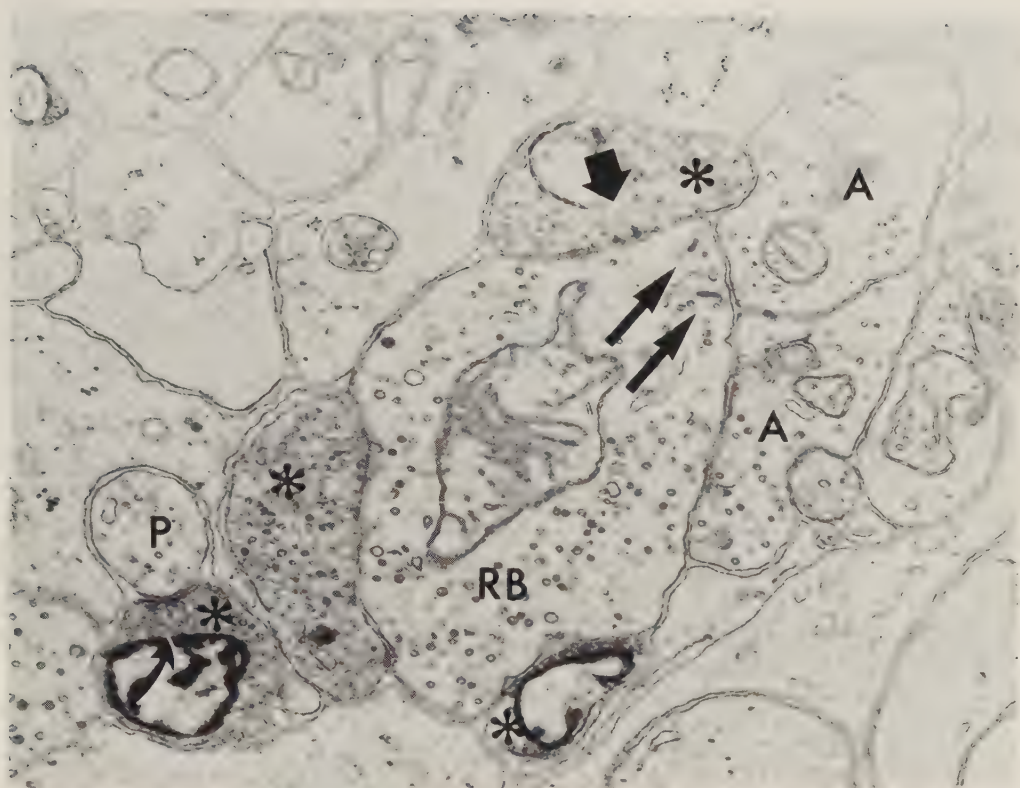


Fig. 6.

Rod bipolar terminal (RB) surrounded by indoleamine-accumulating processes (asterisks) and unlabeled amacrine cell processes (A). There are two ribbon synapses (thin arrows) from the rod bipolar terminal to two dyads of amacrine cell processes. The synapse contacting one labeled and one unlabeled amacrine process is reciprocal in that the labeled process has an output synapse back onto the rod bipolar terminal (thick straight arrow). The labeled process in the lower left corner has an output synapse (curved arrow) to an unidentified process (P). Micrograph of sublamina b_2 of the inner plexiform layer. Mid-peripheral retina. OsO_4 fixation. X 18,500.

Fig. 7.

Tracing from a collage of electron micrographs showing a rod bipolar terminal (RB) and the adjacent neuronal processes in sublaminae b_1 and b_2 in the mid-periphery of an experimental retina. All indoleamine-accumulating processes (shaded grey) surrounding the bipolar terminal are shown. Of the other neuronal processes, only those immediately adjacent to the bipolar terminal are depicted. Four of these processes have not been identified. Empty regions along the rod bipolar terminal are occupied by glial cells. One ribbon synapse in sublamina b_1 , with unlabeled amacrine cell processes (A) in the postsynaptic dyad, is shown as well as three ribbon synapses in sublamina b_2 in dyads consisting of one labeled and one unlabeled amacrine cell process. One of these synapses with a labeled process is reciprocal. There are also two input synapses in sublamina b_2 from labeled processes and one from an unlabeled amacrine process to the rod bipolar terminal. One labeled process in sublamina b_2 has an output synapse to an amacrine cell process which together with a labeled process forms a postsynaptic dyad to the rod bipolar terminal.





Fig. 8.

Labeled process (asterisk) with an output synapse (thick arrow) to an amacrine cell process (A), which has an output synapse (thin arrow) to an unidentified process (P). Sublamina a of the inner plexiform layer. Mid-peripheral retina. OsO_4 fixation. X 45,000.

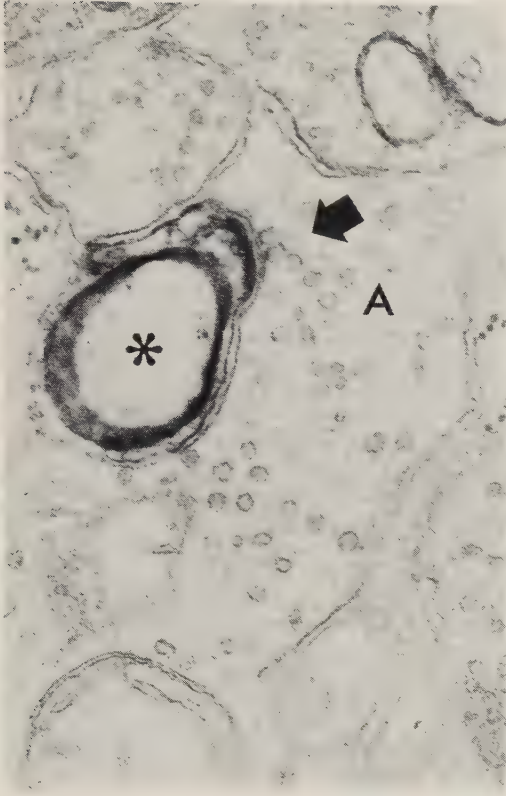


Fig. 9.



Fig. 10.

Labeled process (asterisk) with an input synapse (arrow) from an unlabeled amacrine cell process (A). There is a small cluster of synaptic vesicles on the presynaptic side, and dense material is seen in the synaptic cleft. The dense material on the postsynaptic membrane of the labeled process is prominent. Border region between sublamina a and b₁. Peripheral retina. OsO₄ fixation. X 48,000.

Fig. 10.

Labeled process (asterisk) making synaptic contact (thick arrow) with a ganglion cell dendrite (G), which in turn is post-synaptic to flat cone bipolar process (B). The other postsynaptic element in the dyad is an amacrine cell process (A). The ribbon synapse is marked by a thin arrow. Sublamina a of the inner plexiform layer. Peripheral retina. OsO₄ fixation. X 21,000.



Fig. 11.

Rod bipolar terminal (RB) with a ribbon synapse (thin arrow) onto a dyad consisting of one labeled (asterisk) and one unlabeled (A) amacrine cell process. The unlabeled amacrine cell process of the dyad also receives synaptic input (thick arrows) from two other labeled amacrine cell processes (asterisks). Sublamina b_2 of the inner plexiform layer. Mid-peripheral retina. OsO_4 fixation. X 25,000.

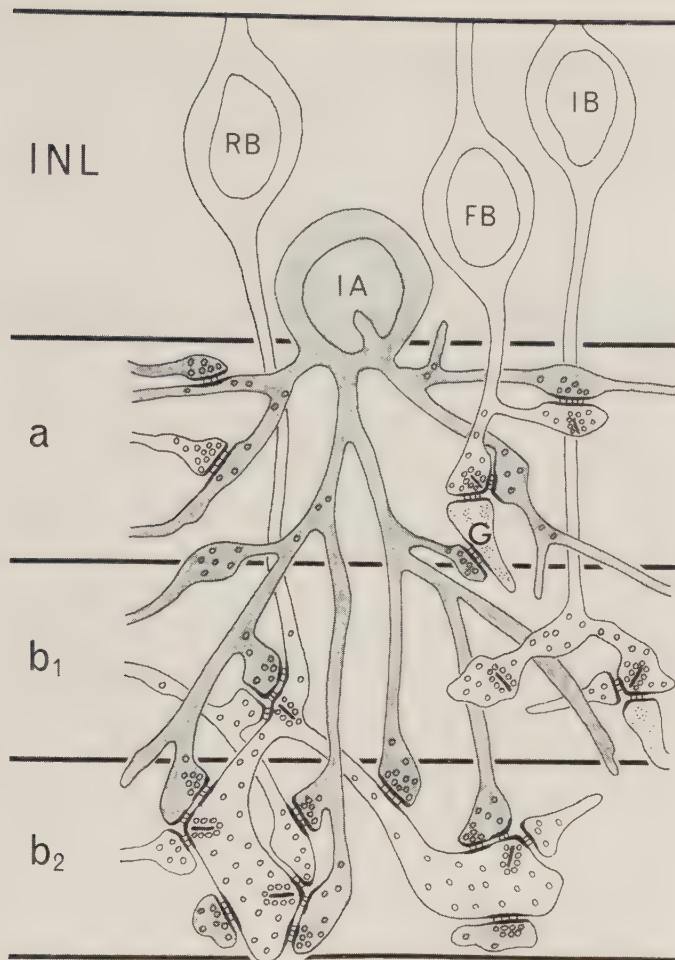


Fig. 12.

Summary diagram of the main synaptic pathways of the indoleamine-accumulating amacrine cells in the retina of the cat. The majority of synaptic contacts are reciprocal synapses with rod bipolar terminals in sublamina b of the inner plexiform layer. Most of these synapses are found in the innermost sublamina, b₂. Four such reciprocal synapses are shown in this diagram as well as three output synapses to rod bipolar terminals. In sublamina a of the inner plexiform layer the indoleamine-accumulating neurons have output and input synapses with flat cone bipolar terminals. There is one synapse of each kind in the diagram. The indoleamine-accumulating processes also exchange synaptic information with other amacrine cell processes throughout the inner plexiform layer. One output synapse to an amacrine cell process is depicted in sublamina b₂. There is an input synapse from an amacrine cell process in sublamina a. There are also synapses between indoleamine-accumulating processes, with one example shown in sublamina a. A few output synapses to ganglion cell dendrites are present. One such output synapse to a ganglion cell dendrite (G) containing only clusters of ribosomes is seen in the border region between sublaminae a and b₁.

SYNAPTIC ORGANIZATION OF THE INDOLEAMINE-ACCUMULATING NEURONS IN
THE CYPRINID RETINA

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ABSTRACT

The distribution and synaptic connections of the indoleamine-accumulating neurons in the retinae of goldfish and carp were studied in the fluorescence and electron microscope.

The indoleamine-accumulating neurons were visualized after the intravitreal injection and uptake of the indoleamine 5,6-dihydroxytryptamine. This labeling procedure produced a characteristic yellow fluorescence of the indoleamine-accumulating neurons and also characteristic ultrastructural changes in them. To avoid interference from the retinal dopaminergic neurons, their processes were either removed by prior treatment with 6-hydroxydopamine or prevented from taking up 5,6-dihydroxytryptamine by the simultaneous injection of the catecholamine alpha-methyl-noradrenaline.

The fluorescence microscopy confirmed earlier reports that the indoleamine-accumulating perikarya and processes are distributed like those of amacrine cells. The indoleamine-accumulating processes ramify in three bands in the inner plexiform layer, the outermost one being the densest.

The electron microscopy showed the indoleamine-accumulating neurons to have synapses of the conventional type, like amacrine cells. Their main synaptic contacts are with other amacrine cells, but synapses with bipolar cell terminals are also present. Both the distribution of the indoleamine-accumulating processes and their synaptic arrangement in the cyprinid retina differ from those found in mammalian retinae earlier investigated.

KEY WORDS: Retina - Synapses - Indoleamine - Electron microscopy - Cyprinid fish

INTRODUCTION

In 1976 Ehinger and Florén described a population of indoleamine-accumulating neurons in the retinae of the rabbit, cat and goldfish. These cells were found to accumulate indoleamines preferentially, a characteristic which made possible their demonstration in the fluorescence microscope with the Falck and Hillarp technique. The neurons did not show any fluorescence in retinae from untreated animals, but after uptake of an exogenous indoleamine such as 5,6-dihydroxytryptamine, they showed the yellow fluorescence typical of indoleamines. Nearly all perikarya of the indoleamine-accumulating neurons were situated among those of the amacrine cells in the inner nuclear layer and their processes ramified in the inner plexiform layer. It was concluded that they constitute a population of amacrine cells.

Indoleamine-accumulating neurons have subsequently been shown to be present to the retina of the Cebus monkey, a New World monkey; in birds such as pigeons and chickens; in amphibians such as mudpuppies and in cyclostomes such as the river lamprey (Dowling and Ehinger, 1975; Ehinger et al., 1977; Florén, 1979; Dowling et al., 1980; Adolph et al., 1980;). In most of these species, the perikarya of the indoleamine-accumulating neurons are found in the innermost cell rows of the inner nuclear layer; but in chickens and pigeons they are usually situated two or three cell rows outwards from the inner plexiform layer. Indoleamine-accumulating perikarya are also seen occasionally in the ganglion cell layer and rarely in the inner plexiform layer in all animals investigated so far. Their processes appear to be confined to the inner plexiform layer in all species with the exception of rare processes, which may extend about halfway into the inner nuclear layer. There are, however, considerable variations between species in the distribution of the indoleamine-accumulating processes within the inner plexiform layer. For example, in the Cebus monkey

and the rabbit the indoleamine-accumulating processes ramify in three bands in the inner plexiform layer and the most vitread band is the densest. In chickens and pigeons there are also three bands but the most sclerad and vitread ones appear to be equally dense, while the middle one is very weak. The mudpuppy displays a rather diffuse ramification of processes in the inner plexiform layer, sometimes with a slightly denser middle band. In the cat, the indoleamine-accumulating processes appear to ramify in several strata in the outer part on the inner plexiform layer and diffusely in the inner part of that layer (Holmgren-Taylor, 1982a). Finally, the carp and goldfish have three bands in the inner plexiform layer, the outermost one being the densest.

Labeling the indoleamine-accumulating neurons with 5,6-dihydroxytryptamine has the advantage of also producing ultrastructural changes in the very same neurons, allowing their identification in the electron microscope. This labeling method has been used to study the synaptic organization of the indoleamine-accumulating neurons in the rabbit, Cebus monkey, mudpuppy and cat (Ehinger and Holmgren, 1979; Dowling *et al.*, 1980; Adolph *et al.*, 1980; Holmgren-Taylor, 1982a). These electron microscopical investigations have all shown that the indoleamine-accumulating neurons have synapses of the conventional type with a presynaptic cluster of small synaptic vesicles. Such synapses are typical of amacrine cells, providing additional evidence that the indoleamine-accumulating neurons form a population of amacrine cells.

The synaptic contacts of the indoleamine-accumulating neurons have been most extensively investigated in the rabbit and the cat (Ehinger and Holmgren, 1979; Holmgren-Taylor, 1982a). In both these mammals the main connections are reciprocal synapses with presumably rod bipolar terminals in the innermost part of the inner plexiform layer. There is a smaller number of synapses with other amacrine cells, and in the cat also with ganglion cells. The study of the Cebus monkey indicates a similar predominance of

synapses with bipolar terminals in the innermost part of the inner plexiform layer (Dowling et al., 1980).

The physiology and morphology of the teleost retina have been extensively studied, but information is not available on the synaptic contacts made by its indoleamine-accumulating neurons. The aim of the present investigation has been therefore, to study the synaptic organization of the indoleamine-accumulating neurons in the retina of goldfish and carp and to compare it with that of the indoleamine-accumulating neurons in the other species already investigated.

Since the dopaminergic retinal neurons also accumulate 5,6-dihydroxytryptamine and since this accumulation, apart from enhanced fluorescence, also causes ultrastructural changes in them (Ehinger et al., 1969; Dowling and Ehinger, 1975 and 1978a), it is necessary to prevent the labeling of the dopaminergic processes before the analysis of the indoleamine-accumulating processes can be carried out. One way is to destroy selectively the dopaminergic processes. A method for this, using 6-hydroxydopamine, is available (Ehinger and Nordenfelt, 1977) and has been used in most experimental eyes in this study. Another way is to prevent the dopaminergic processes from taking up 5,6-dihydroxytryptamine. This is achieved through the simultaneous injection of both the labeling drug and alpha-methyl-noradrenaline, a catecholamine, which is accumulated preferentially by the dopaminergic neurons (Ehinger and Florén, 1976). This method has been used previously in fluorescence microscopic studies, and is shown here to work also for electron microscopic studies.

MATERIALS AND METHODS

Adult (5-15 cm) carp (Cyprinus carpio) and goldfish (Carassius auratus) were given intravitreal injections of 1, 1.5, 5 or 10 ug 6-hydroxydopamine on two consecutive days in order to remove the dopaminergic processes (Ehinger and Nordenfelt, 1977). One week later these experimental eyes were given injections of 1, 2.5, 5 or 10 ug 5,6-dihydroxytryptamine intravitreally, and they were then enucleated 4 hours later. The control eyes were enucleated one week after the 6-hydroxydopamine injections.

A smaller number of experimental eyes were just given one intravitreal injection of 1, 2, 5 or 10 ug alpha-methyl-noradrenaline and 5 or 10 ug 5,6-dihydroxytryptamine 4 hours before enucleation. At least two eyes were used for each drug combination, except for the alpha-methyl-noradrenaline and 5,6-dihydroxytryptamine eyes, of which only one eye was used for each combination.

After enucleation, the eyes were incised at the ora serrata and the anterior segments were cut away and discarded. Pieces from both experimental and control posterior segments were processed for fluorescence microscopy of biogenic monoamines according to the method of Falck and Hillarp (see Björklund et al., 1972). Retinal sections were then viewed in the fluorescence microscope with a barrier filter with its 50% cut-off edge at about 500 nm. This allowed the selection for electron microscopy of only well-labeled retinæ with complete degeneration of the dopaminergic processes in the inner plexiform layer.

The remaining pieces of the posterior segments of approximately half of the experimental eyes were fixed for an hour in ice cold 2.5% glutaraldehyde in 65 mM cacodylate buffer with 4.5 mM CaCl_2 and washed for 5 minutes in ice cold 67 mM cacodylate buffer with 0.13 M sucrose. They were then postfixed in 2% OsO_4 in veronal acetate buffer (pH 7.6-7.8) containing 28 mM barbital sodium and 28 mM sodium acetate with 1.8 mM CaCl_2 and 66 mM

sucrose for an hour in an ice bath, and half an hour at room temperature. The remaining pieces of the other half of the experimental eyes were only fixed in OsO_4 . After a 5 minute wash in veronal acetate buffer, there followed dehydration in graded alcohols and propylene oxide and finally embedding in Epon 812. Ultra-thin sections were stained with uranyl acetate and lead citrate and analyzed in Philips 300 and Jeol 100B electron microscopes.

6-hydroxydopamine hydrobromide and 5,6-dihydroxytryptamine creatinine sulfate were obtained from Regis Chemical Co., Morton Grove, Illinois, USA, and alpha-methyl-noradrenaline, Corbasil (R), from Hoechst Laboratories, Mölndal, Sweden. (1 ug alpha-methyl-noradrenaline corresponds to about 2 ug 5,6-dihydroxytryptamine on a molar basis). All weights given refer to the salts. All drugs were dissolved in 0.9% NaCl with ascorbic acid (1 mg/ml) added as anti-oxidant.

RESULTS

Light microscopy

When untreated retinæ of cyprinid fish are processed according to the Falck and Hillarp method for fluorescence microscopy of structures containing biogenic monoamines, only the green fluorescing cell bodies and processes of the dopaminergic interplexiform cells are seen (Ehinger *et al.*, 1969; Dowling and Ehinger, 1975 and 1978a). After a single injection of 5,6-dihydroxytryptamine both the dopaminergic and the indoleamine-accumulating neurons are visualized in the fluorescence microscope (Ehinger and Florén, 1976). It is often possible to distinguish between the perikarya of the two cell populations since the dopaminergic neurons tend to fluoresce greenish-yellow and the indoleamine-accumulating neurons have the characteristic yellow fluorescence of indoleamines. It is much more difficult, however, to distinguish the greenish-yellow dopaminergic processes from the yellow indoleamine-accumulating ones. After the simultaneous injection of alpha-methyl-noradrenaline, which is accumulated preferentially by the dopaminergic

neurons, and 5,6-dihydroxytryptamine, which is accumulated preferentially by the indoleamine-accumulating neurons, it is usually possible to distinguish between both the perikarya and the processes of the two cell populations. The dopaminergic neurons fluoresce green and the indoleamine-accumulating ones fluoresce yellow (Ehinger and Florén, 1976). With 6-hydroxydopamine treatment prior to the 5,6-dihydroxytryptamine injection, nearly all of the dopaminergic processes are destroyed, leaving only the yellow fluorescing indoleamine-accumulating ones (Ehinger and Nordenfelt, 1977). The dopaminergic perikarya remain, however, displaying a greenish-yellow fluorescence among the yellow indoleamine-accumulating perikarya.

From the examination of both types of experimental retinae and the 6-hydroxydopamine treated control retinae, it is seen that the majority of indoleamine-accumulating perikarya is characteristically situated one or two cell rows outwards in the inner nuclear layer (Fig. 1). In contrast, the dopaminergic perikarya are typically found in the innermost cell row of the inner nuclear layer. Both kinds of neurons occasionally have cell bodies in the ganglion cell layer and rarely in the inner plexiform layer. Furthermore, the indoleamine-accumulating perikarya are smaller in size than the dopaminergic ones; and by a rough estimate they are at least twice as many as the dopaminergic perikarya in the inner nuclear layer.

The indoleamine-accumulating processes ramify within the inner plexiform layer (Fig. 1). Three strata of processes can be distinguished, one in each of the innermost, middle and outermost parts of the inner plexiform layer; roughly corresponding to Ramon y Cajal's stratum 1, 3 and 5 (Ramon y Cajal, 1972). Of these, stratum 1 is the densest one. The number of indoleamine-accumulating processes in stratum 3 is much less and there are even fewer in stratum 5. This distribution of the indoleamine-accumulating processes in the inverse of that of the dopaminergic ones, which

are most numerous in the inner parts of the inner plexiform layer and fewer in the outer parts. No indoleamine-accumulating processes are seen to extend more than halfway into the inner nuclear layer or to reach the outer plexiform layer.

Some green fluorescing processes of the dopaminergic interplexiform cells are occasionally left in the outer plexiform and inner nuclear layers of the 6-hydroxydopamine treated retinae. High doses of 6-hydroxydopamine may induce generalized damage to the retina (Ehinger and Nordenfelt, 1977). Thus, it is not desirable to increase the doses of 6-hydroxydopamine above those which successfully destroy the dopaminergic process in the inner plexiform layer. No 6-hydroxydopamine treated experimental retinae with demonstrable green fluorescing dopaminergic processes remaining in the inner plexiform layer have been used in this study.

Electron microscopy

1. Appearance of labeled neurons

The labeled indoleamine-accumulating processes have increased electron density of their cell membranes and the membranes of cell organelles such as mitochondria, microtubules and small synaptic vesicles of about 40-50 nm diameter (Figs. 2, 3 and 4). The increased density of the cell membrane is often patchy. The denser regions of the membrane sometimes have electron dense material on both the cytoplasmic and extracellular sides even when there are no signs of any synapse. The mitochondria are swollen and their cristae have often disappeared, giving them an empty appearance (Fig. 4). The small synaptic vesicles are sometimes flattened in shape and occasionally contain an electron dense eccentric spot (Fig. 2). Large dense-cored synaptic vesicles have increased electron density of the central core, and these vesicles are often also distorted.

The labeled processes often appear dense and are filled with tightly packed small synaptic vesicles and mitochondria (Fig. 3). Other labeled processes have a more translucent appearance with a few scattered aggregations of small synaptic vesicles and mitochondria in an empty cytoplasm (Figs. 4 and 5).

Labeled indoleamine-accumulating perikarya can be distinguished from the dopaminergic ones because of their outward position and their small size. The indoleamine-accumulating perikarya are also about twice as common as the dopaminergic ones. The labeled perikarya have degenerative changes in both nucleus and cytoplasm (Fig. 6). The normal granular chromatin pattern of the nucleus is broken and there are electron-dense aggregations of material scattered in the nucleoplasm. In the cytoplasm there are aggregations of ribosome-like particles with increased electron density, and dilated vesicular structures, most likely representing rough and smooth endoplasmic reticulum.

There is a variability in the ultrastructural changes produced by the labeling procedure both among retinæ and within any particular retina. This variability is in part a dosage effect; the larger doses of 5,6-dihydroxytryptamine cause increased disorganization of the normal cytoarchitecture in each group of fish of the same size.

All the retinæ used in this analysis have a normal morphology apart from the ultrastructural changes in the labeled neurons. Only extremely rarely can a dense process with severe degenerative changes be seen in the control retinæ subjected to the 6-hydroxydopamine treatment only. These processes are considered to be remaining dopaminergic ones. The appearance of the labeled processes is the same both when the injection of 5,6-dihydroxytryptamine is preceded by 6-hydroxydopamine treatment and when the labeling drug is given simultaneously with alpha-methyl-noradrenaline. The distribution of the characteristically labeled processes

as well as the distribution and appearance of their synaptic contacts are also similar in both sets of experimental retinae. In the retinae treated with alpha-methyl-noradrenaline and 5,6-dihydroxytryptamine, there are occasionally processes that contain small synaptic vesicles with an eccentric dense spot and large dense-cored vesicles with increased density of the core. These processes are seen both in the outer and inner plexiform layers. They do not have any changes in other cell organelles nor any increased density of the cell membrane. Their distribution in the inner plexiform layer is similar to that of the dopaminergic interplexiform cell processes. They are considered to be dopaminergic processes, and the inconspicuous ultrastructural changes in them are either caused by alpha-methyl-noradrenaline or by small amounts 5,6-dihydroxytryptamine accumulated along with the preferred catecholamine. This inconspicuous labeling is detected in the electron microscope only with careful searching at high magnification, and it is easily distinguished from the pronounced labeling of the indoleamine-accumulating neurons.

2. Distribution of labeled processes

The labeled processes are beaded; thin intervaricose segments alternate with mainly small to medium-sized varicosities (Fig. 3). The distribution of labeled processes seen in the electron microscope is in agreement with the distribution of processes seen in the fluorescence microscope. Labeled indoleamine-accumulating processes have only been found in the inner plexiform layer.

With high doses of 5,6-dihydroxytryptamine, generally more than 5 ug, a small subpopulation of labeled processes with a specific distribution appears. They are all found in stratum 3 of the inner plexiform layer. Their varicosities are much larger than the other labeled ones and appear more swollen (Fig. 7). The ultrastructural changes are similar to those earlier described, but these varicosities usually have an empty-looking cytoplasm with scattered clusters of small synaptic vesicles and swollen

mitochondria. In the retinae treated with alpha-methyl-noradrenaline the increase in density of the cell membrane and cell organelles in the large labeled processes is less than in the other labeled processes. They have both conventional amacrine output synapses and a previously not recognized type of junction with two postsynaptic processes. This type of junction, which has been called a branched conventional synapse, is further described below and in more detail elsewhere (Holmgren-Taylor, 1982b).

3. Appearance of junctional regions

All labeled processes make synapses of the kind typical of amacrine cells (Dowling and Boycott, 1966; Witkovsky and Dowling, 1969). On the presynaptic side, there is a cluster of small synaptic vesicles close to the presynaptic membrane and some electron dense depositions on that membrane; which itself also has increased density. The synaptic cleft contains electron dense fibrillar material and there is usually both increased electron density of the postsynaptic membrane and some flocculent or fibrillar material deposited on it (Figs. 2, 4 and 8). When postsynaptic, the indoleamine-accumulating processes usually display a pronounced electron density of the cell membrane in the synaptic region and some dense material deposited on the membrane (Figs. 2 and 9).

Junctions between two labeled indoleamine-accumulating processes are desmosome-like with symmetric depositions of dense material on the junctional membrane of both processes and electron dense material in the intercellular space. There are also desmosome-like junctions between labeled and unlabeled neuronal processes.

The large processes of stratum 3 of the inner plexiform layer have output synapses of the conventional type, but they also often have a special type of synapse, the branched conventional synapse (Holmgren-Taylor, 1982b). In these synapses, there is a slight protrusion on the part of the indoleamine-accumulating process

juxtaposed to two neuronal processes. There is a small cluster of 40-50 nm synaptic vesicles in the protrusion, often around a central dense globule. The cell membrane of the protrusion has dense material on it, and the intercellular space between the indoleamine-accumulating process and the two adjoining neuronal processes is filled with dense material. The two other processes also have dense, flocculent or fibrillar depositions on their cell membranes in the synaptic region (Fig. 7).

4. Synaptic connections

The density of indoleamine-accumulating processes and synapses in the goldfish retina is low compared to, for example, the rabbit. However, about 200 synapses have been observed in random single sections and short series of consecutive sections. Table 1 summarizes the kinds of synaptic contacts found in a sample of 150 synapses. The indoleamine-accumulating neurons have synaptic connections mainly with other amacrine cell processes. Input synapses from amacrine cell processes are very common. These synapses are found in all three bands of the inner plexiform layer in proportion to the number of indoleamine-accumulating processes ramifying there. They involve both the intervaricose segments and the varicosities of the indoleamine-accumulating neurons, including the varicosities of the middle band (Figs. 2 and 9). Two suspected branched conventional synapses have also been detected, where one of the two postsynaptic elements is a large labeled process of the special type.

The number of output synapses onto other amacrine cells is smaller, but also roughly proportional to the number of labeled processes in each of the three bands. The postsynaptic element can be either an amacrine cell varicosity or an intervaricose segment (Fig. 8). One output synapse onto a perikaryon in the innermost cell row of the inner nuclear layer has been detected (Fig. 2); suggesting that synapses to amacrine cell perikarya occur but are not very common. The majority of the unidentified postsynaptic

elements (? in Table 1) is believed to be amacrine cell processes. The special large varicosities in the middle third of the inner plexiform layer have a few conventional output synapses onto other amacrine cell processes, but the majority of their junctions with amacrine cell processes are of the branched conventional type, and consequently involve two different postjunctional processes. These are usually two amacrine cell processes or one amacrine cell process and one unidentified process (Fig. 7). No bipolar terminal has been among the postsynaptic elements in the branched conventional synapses of the large indoleamine-accumulating processes.

A small number of synaptic contacts with bipolar terminals with synaptic ribbons and an even smaller number of contacts with suspected bipolar processes have been found. Five output synapses onto bipolar processes with obvious synaptic ribbons have been seen in the sclerad band of the inner plexiform layer (Figs. 4 and 5). These bipolar terminals range in size from large to small. One output synapse has been detected onto a medium-sized bipolar terminal in the inner part of the inner plexiform layer. Four output synapses onto suspected bipolar terminals in the sclerad and middle bands of the inner plexiform layer involve large and medium-sized bipolar terminals.

Three input synapses onto labeled processes from bipolar terminals have been seen; two in the sclerad band and one in the vitread band. The other postsynaptic elements of the dyads are, in all three cases, unlabeled amacrine cell processes. The bipolar terminals are of varying size. Of the output and input synapses with bipolar terminals now mentioned, two represent reciprocal synapses. One of these has been detected in the vitread band and the other in the sclerad one, and the bipolar terminals have been large and medium-sized, respectively. The large processes of the middle band have not been found to engage in synapses of the conventional type with verified or suspected bipolar terminals.

No synapses with processes identified as ganglion cell dendrites have been seen, though some contacts with suspected ganglion cell dendrites have been seen in all three bands of the inner plexiform layer. Serial section analysis has so far failed to show any synaptic contacts between indoleamine-accumulating processes and the unmyelinated terminals of the centrifugal fibres which have been identified by tracing them to their myelinated preterminals (Witkovsky, 1971). No synapses have been detected between two indoleamine-accumulating processes.

A variety of serial synaptic arrangements involving labeled processes has been found. Labeled processes have output synapses onto both the varicosities and intervaricose segments of amacrine cell processes which in turn make output synapses onto other amacrine cell processes or bipolar terminals (Fig. 8). Amacrine cell processes have output synapses onto labeled processes which in turn have output synapses onto unidentified processes or as in one case, onto a cell body in the innermost cell row of the inner nuclear layer (Fig. 2). A bipolar terminal has also been seen with a dyad output synapse onto a labeled process and an amacrine cell process which in turn made a synapse onto the labeled process of the same dyad.

DISCUSSION

The distribution of indoleamine-accumulating perikarya and processes found in this investigation both in the fluorescence microscope and the electron microscope is in agreement with the findings of Ehinger and Florén (1976). The present electron microscopic analysis also confirms their assumption that the indoleamine-accumulating neurons in the goldfish form a population of amacrine cells; since not only the localization of the indoleamine-accumulating perikarya to the innermost part of the inner nuclear layer and the ramification of their processes in the

inner plexiform layer but also their conventional type of synapses are characteristic of amacrine cells.

The ultrastructural changes produced in the indoleamine-accumulating neurons by 5,6-dihydroxytryptamine in this investigation are similar to those seen in earlier studies of indoleamine-accumulating neurons in other animal species (Ehinger and Holmgren, 1979; Dowling *et al.*, 1980; Adolph *et al.*, 1980; Holmgren-Taylor, 1982a). The changes seen are thought to be due in part to the accumulation of the osmiophilic labeling drug in mainly the synaptic vesicles of the neurons and in part to the neurotoxic effect of the dihydroxylated indoleamine (Baumgarten and Björklund, 1976). This neurotoxicity explains the increased disruption of the normal ultrastructure of the labeled neurons with larger doses of 5,6-dihydroxytryptamine. Asymmetry of injection of the labeling drug and perhaps also different states of activity of the labeled cells presumably also account for the variability in the labeling.

High doses of 5,6-dihydroxytryptamine bring out a special small subset of indoleamine-accumulating processes with characteristically large varicosities, localization limited to the middle band in the inner plexiform layer, and a special type of synapse, the branched conventional synapse (Holmgren-Taylor, 1982b). This is phenomenon not seen in the other species investigated. With very large doses of 5,6-dihydroxytryptamine it is known that, for instance in the avian retina, bipolar-like neurons show indoleamine fluorescence in addition to the indoleamine-accumulating amacrine cells (Florén, 1979). However, the distribution of fluorescing perikarya and processes in the fluorescence microscope appears to be the same both when the large indoleamine-accumulating processes are found in the electron microscope, and when the large processes cannot be detected. The special large processes have not been seen to contain synaptic ribbons of the bipolar type. Furthermore, the increase in the dose of 5,6-dihydroxytrypt-

amine needed to bring out this subset of indoleamine-accumulating neurons does not seem to affect the labeling of the other processes. The special large processes are similar to the other indoleamine-accumulating processes in that they have both output and input synapses of the conventional type with other amacrine neurons. However, they also have branched conventional synapses. The possibility cannot be excluded that these special processes belong to subpopulation of indoleamine-accumulating amacrine cells with a somewhat less effective uptake mechanism for 5,6-dihydroxytryptamine or a greater resistance against the toxic effects of the drug. However, other explanations cannot be ruled out. For instance, the functional state of the indoleamine-accumulating neurons under the experimental conditions could perhaps cause certain parts of the dendritic tree to have a different reaction pattern to 5,6-dihydroxytryptamine.

The experimental results are to some extent dependent on the reliability of the removal of the dopaminergic neurons with 6-hydroxydopamine. It is therefore important that the alternative procedure, treating the retinae with both alpha-methyl-noradrenaline and 5,6-dihydroxytryptamine, produces only mild labeling in the dopaminergic neurons and strong labeling in the indoleamine-accumulating neurons. With this procedure, the results are identical with those seen after the 6-hydroxydopamine treatment; corroborating the description given.

The synaptic pathways of the indoleamine-accumulating neurons in the cyprinid retina are summarized in Fig. 10. The indoleamine-accumulating neurons mainly form interamacrine connecting links. Since the majority of indoleamine-accumulating processes ramify in the outer third of the inner plexiform layer, this is where the majority of synapses with other amacrine cells are found. The contacts with bipolar cells are fewer. The indoleamine-accumulating neurons are both pre- and postsynaptic to bipolar cells and also form reciprocal synapses with them. The postsynaptic partners

in the output dyads so far seen have all been amacrine cell processes. Bipolar contacts are present in all three bands of the inner plexiform layer, and furthermore, the bipolar terminals so far observed have varied considerably in size, which may mean that both cone bipolar cells and several types of rod-cone bipolar cells are involved (Ishida et al., 1980).

The synaptic organization of the indoleamine-accumulating neurons in the cyprinid retina differs significantly from that of the indoleamine-accumulating neurons in the other species investigated. In cyprinid fish, the indoleamine-accumulating neurons predominantly form interamacrine links. In contrast, in the rabbit, Cebus monkey and cat the majority of their synapses are with bipolar cells, presumably mainly rod bipolar cells, in the innermost part of the inner plexiform layer. The predominant synaptic arrangement with these bipolar cells seems to be reciprocal contacts (Ehinger and Holmgren, 1979; Dowling et al., 1980; Holmgren-Taylor, 1982a). These species differences in the synaptic pathways of the indoleamine-accumulating neurons can be compared to those known for the dopaminergic retinal neurons. In teleost fish and New World monkeys they are interplexiform cells (Laties and Jacobowitz, 1966; Ehinger et al., 1969; Ehinger and Falck, 1969; Dowling and Ehinger, 1975 and 1978a; Dowling et al., 1980). In the rabbit, Cynomolgus monkey and cat, however, the dopaminergic neurons are amacrine cells forming an interamacrine network (Dowling and Ehinger, 1978b; Pourcho, 1981; Holmgren, 1982).

The majority of the synapses made by the indoleamine-accumulating neurons in the cyprinid retina is found in the outer half of the inner plexiform layer, often designated sublamina a. Functionally this layer mediates OFF-signals from bipolar cells to OFF-ganglion cells, whose dendrites terminate in this sublayer (Famiglietti et al., 1977). The inner half of the inner plexiform layer, designated sublamina b, similarly mediates ON-signals from bipolar cells to ON-ganglion cells, whose dendrites ramify in that

sublayer. This may suggest that the indoleamine-accumulating cells in the cyprinid retina are mainly involved in the OFF-pathway. It is, however, at the same time clear that the presence of synaptic contacts - even if fewer in number - in sublamina b indicates a more complex interconnective role between the ON- and OFF-pathways for the indoleamine-accumulating cells.

It is not yet known if the indoleamine-accumulating neurons have any connections with the other amacrine cell types whose synaptic organization has been characterized on the basis of their putative transmitters. In this context both the glycine- and the GABA-accumulating neurons (Marc et al., 1978; Marc and Lam, 1981) may be of interest, since there is at least partial overlap of their dendritic ramifications with those of the indoleamine-accumulating cells. All three types of amacrine cells do, however, have their densest dendritic bands in different sublayers in the inner plexiform layer, namely the indoleamine-accumulating ones in stratum 1; the glycine-accumulating ones in strata 2-3 and the GABA-accumulating ones in strata 3-5. Furthermore, the dopaminergic interplexiform cells must be considered as possible synaptic partners. They are pre- and postsynaptic to amacrine cell processes in the inner plexiform layer, even though their dendrites mainly ramify in the innermost third of the inner plexiform layer (Dowling and Ehinger, 1975 and 1978a). However, more information is needed about the synaptic contacts of these different cell populations before any inferences can be made about how the neurons with identified transmitters interact.

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Table 1

Synapses made by the indoleamine-accumulating neurons
in the cyprinid retina

Inner plexiform layer				
Strata:	1	3	5	
OUTPUT TO:				
Amacrine	23	4	2	
Bipolar	7	2	1	
Ganglion	0	0	0	
?	20	16	8	
INPUT FROM:				
Amacrine	41	19	0	
Bipolar	2	1	1	
RECIPROCAL:				
Amacrine	0	0	0	
Bipolar	1	0	1	

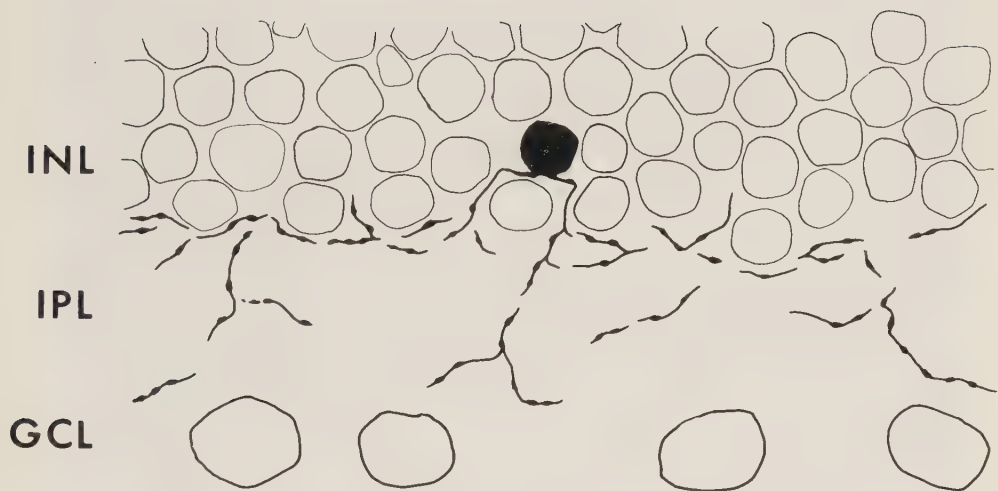


Fig. 1.

Semi-diagrammatic drawing of the localization of indoleamine-accumulating cell bodies and processes seen in the fluorescence microscope. A relatively small cell body is situated in the second cell row of the inner nuclear layer. Indoleamine-accumulating processes form a network in the outermost stratum of the inner plexiform layer. A few processes extend deeper into the middle and innermost strata and ramify there. GCL = ganglion cell layer, INL = inner nuclear layer and IPL = inner plexiform layer.

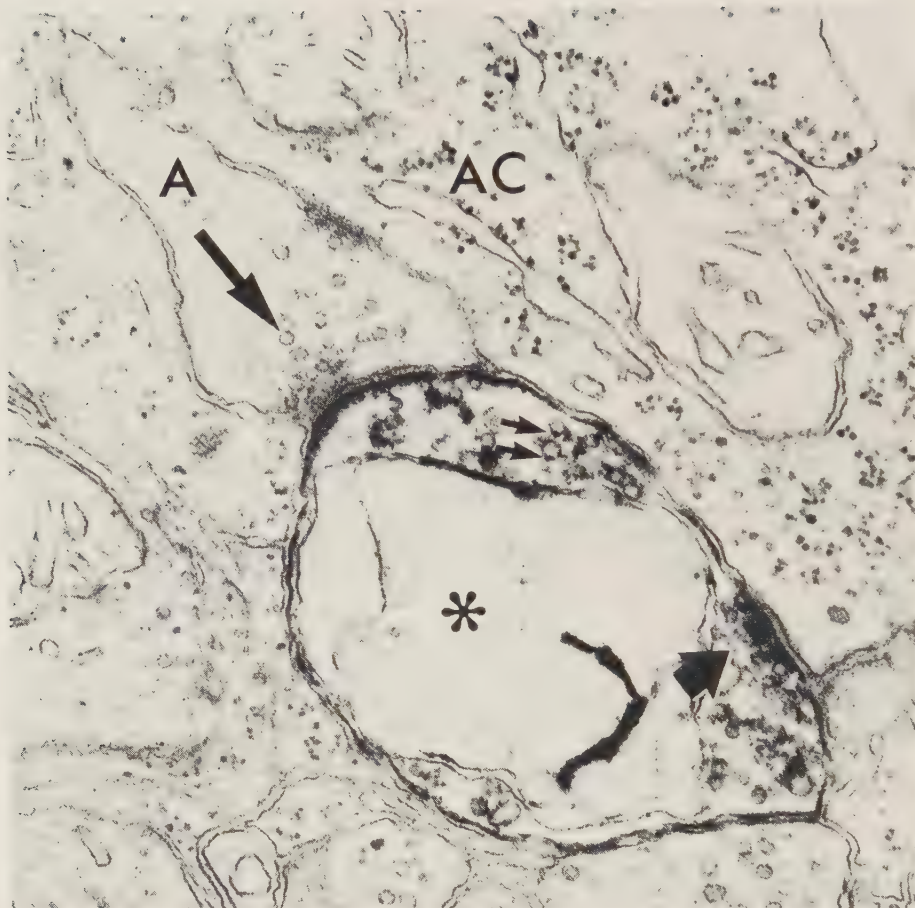


Fig. 2.

Labeled process (asterisk) with input synapse (thin arrow) from unlabeled amacrine cell process (A) and output synapse (thick arrow) to a perikaryon (AC) in the innermost cell row of the inner nuclear layer. The labeled process contains a swollen distorted mitochondrion, and small synaptic vesicles, some of which display an eccentric electron dense spot (small arrows). Both synapses are of the conventional kind with a presynaptic cluster of small synaptic vesicles, pre- and postsynaptic membrane densities (prominent in the labeled process) and dense material in the synaptic cleft. Retina of carp treated with 1.5 ug 6-hydroxydopamine and 1 ug 5,6-dihydroxytryptamine. OsO_4 fixation. X 30,000



Fig. 3.

Labeled indoleamine-accumulating process (asterisk) with a varicosity (v) and its connecting intervaricose segment (ivs). The cell membrane as well as microtubules in the intervaricose segment and small synaptic vesicles and vesicular structures in the varicosity show increased electron density. Stratum 3 of the inner plexiform layer. Electron micrograph of the retina of a carp injected intravitreally with 10 ug alpha-methyl-noradrenaline and 10 ug 5,6-dihydroxytryptamine. OsO_4 fixation. X 44,000

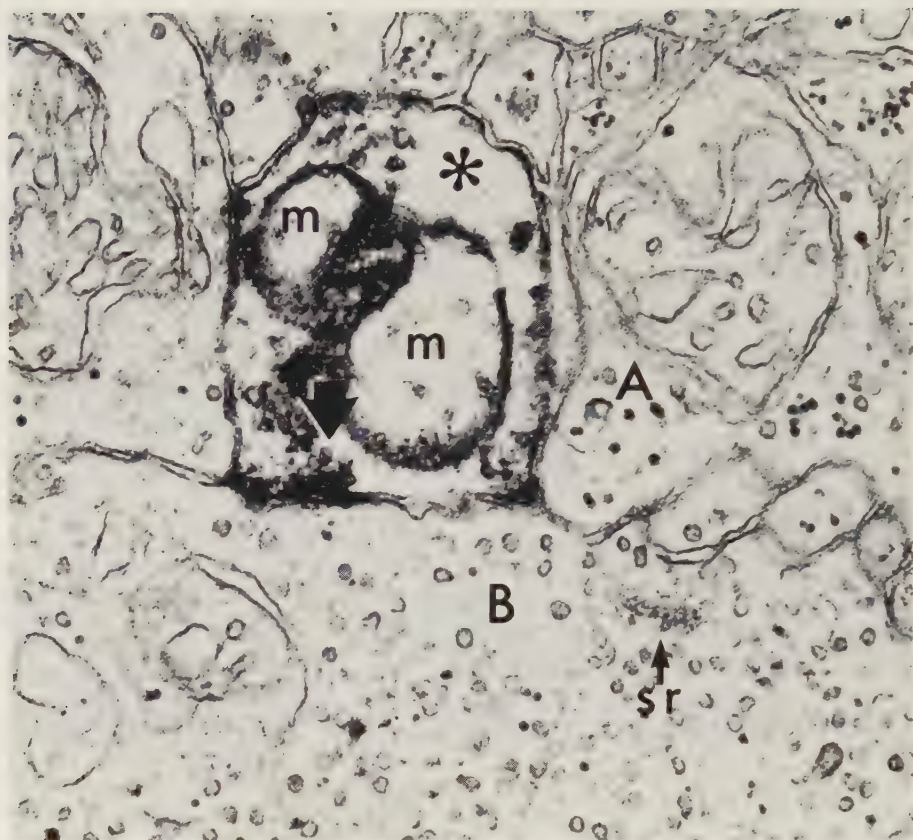


Fig. 4.

Output synapse (thick arrow) from a labeled process (asterisk) onto a large bipolar terminal (B) in stratum 1 of the inner plexiform layer. The synaptic ribbon (sr) in the bipolar terminal was unequivocal in subsequent sections. Dense mitochondria in the labeled process are marked m. An adjacent amacrine cell process is marked A. Retina of a carp injected with 10 ug alpha-methylnoradrenaline and 10 ug 5,6-dihydroxytryptamine. OsO_4 fixation. X 49,000

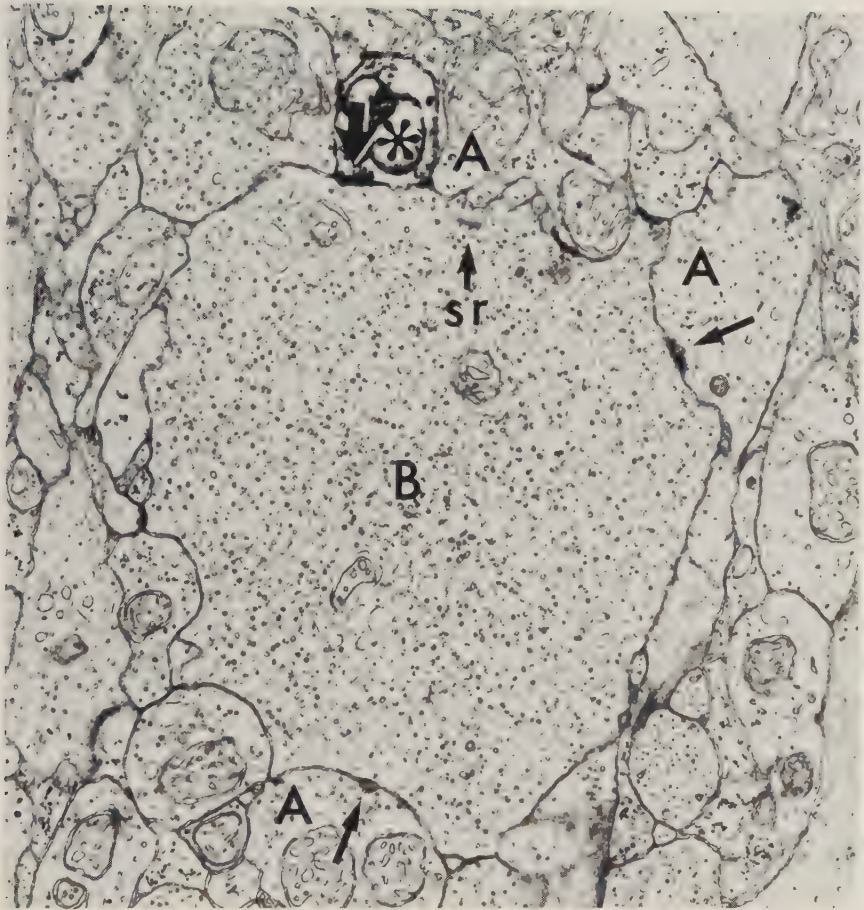


Fig. 5.

Lower magnification electron micrograph of the processes and the synapse (thick arrow) in Fig. 4. The labeled process (asterisk) is easy to distinguish from the unlabeled processes because of the increased density of its cell membrane and cell organelles. The labeled process is of the translucent variety with scattered clusters of small synaptic vesicles and mitochondria. Unlabeled amacrine cell processes with output synapses (thin, long arrows) to the bipolar terminal are marked A. X 19,000

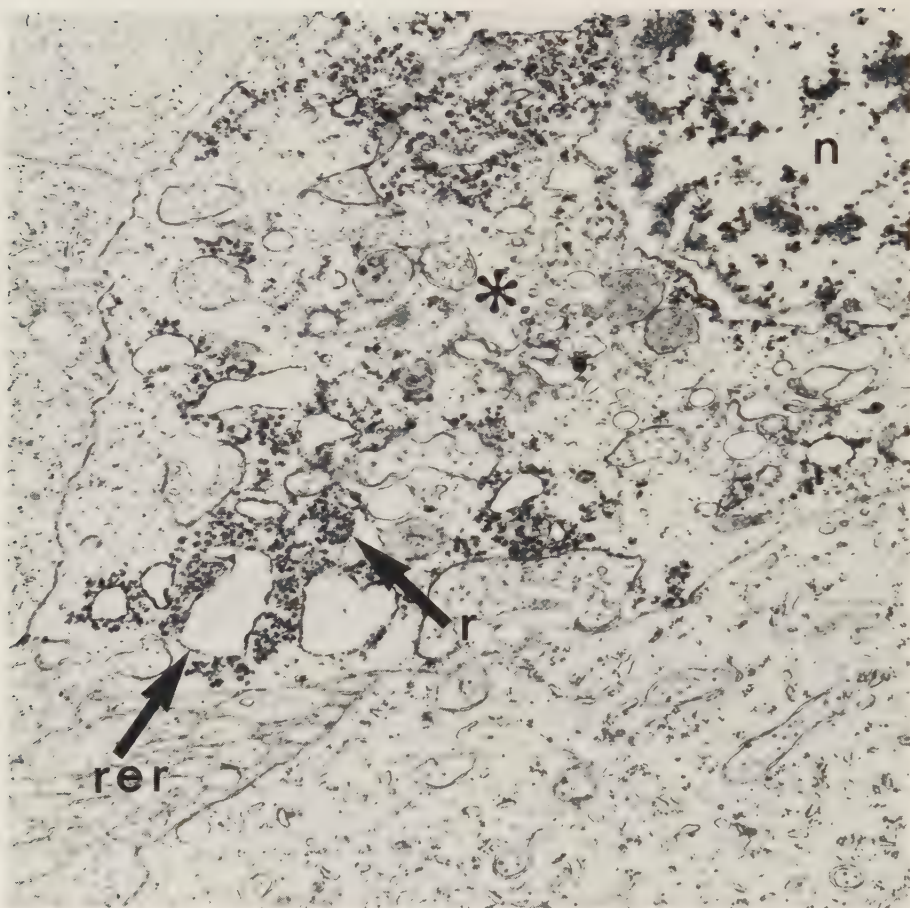


Fig. 6.

Labeled small perikaryon (asterisk) in the second cell row of the inner nuclear layer. The nucleoplasm (n) contains aggregations of electron dense material. In the cytoplasm there are clusters of ribosome-like particles (r) and dilated endoplasmic reticulum (rer). The cytoplasm of adjacent perikarya is shown for comparison. Retina of a carp, injected with 10 ug alpha-methylnoradrenaline and 10 ug 5,6-dihydroxytryptamine. OsO₄ fixation. X 13,000

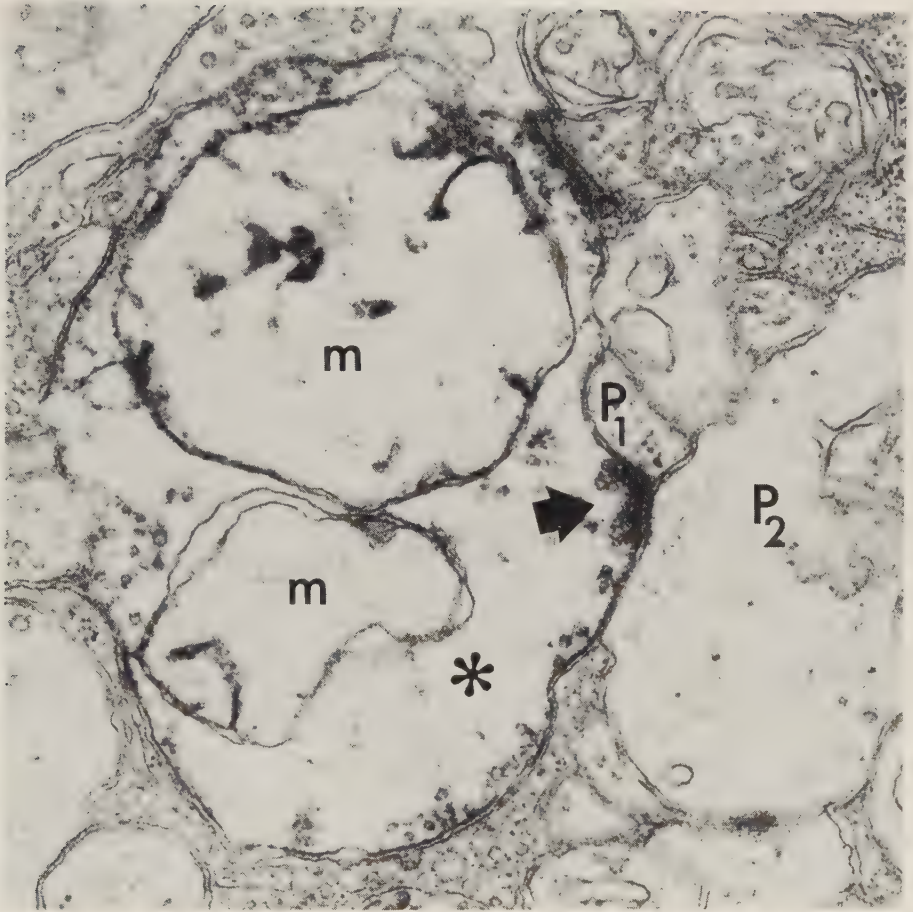


Fig. 7.

Large labeled varicosity (asterisk) with a branched conventional synapse (thick arrow) to two other neuronal processes (P_1 and P_2) in stratum 3 of the inner plexiform layer. The varicosity appears swollen with likewise swollen mitochondria and scattered clusters of small synaptic vesicles. There is a presynaptic cluster of small synaptic vesicles in a protrusion of the varicosity, electron dense material in the intercellular space between the labeled varicosity and the other two processes as well as dense material on the postsynaptic membranes of the other processes. m = mitochondrion. Retina of a goldfish, treated with 5 ug 6-hydroxydopamine and 10 ug 5,6-dihydroxytryptamine. OsO_4 fixation. X 45,000

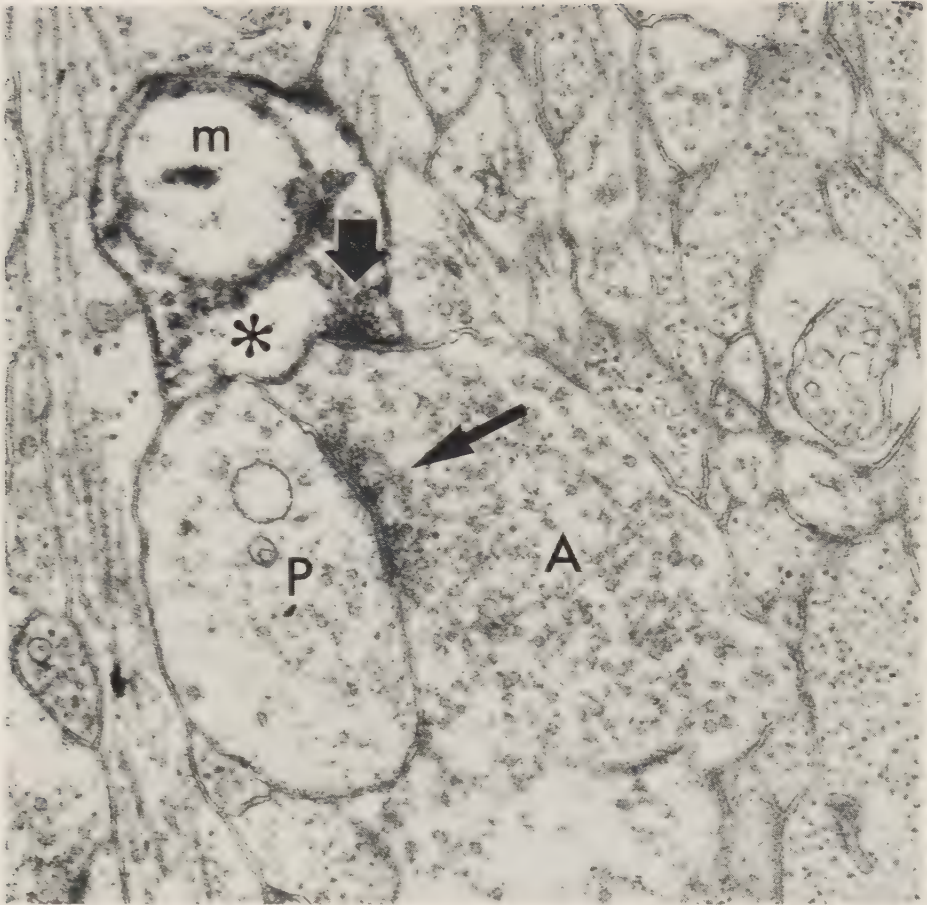


Fig. 8.

Labeled varicosity (asterisk) with output synapse (thick arrow) to an unlabeled amacrine cell varicosity (A) with output synapse (thin arrow) to an unidentified neuronal process (P). Both are conventional synapses with presynaptic clusters of small synaptic vesicles. There is dense material on the presynaptic membranes (most prominent in the labeled process), in the synaptic clefts and on the postsynaptic membranes. m = mitochondrion. Stratum 1 of the inner plexiform layer. Retina of a goldfish treated with 10 ug 6-hydroxydopamine and 10 ug 5,6-dihydroxytryptamine. OsO_4 fixation. X 42,000

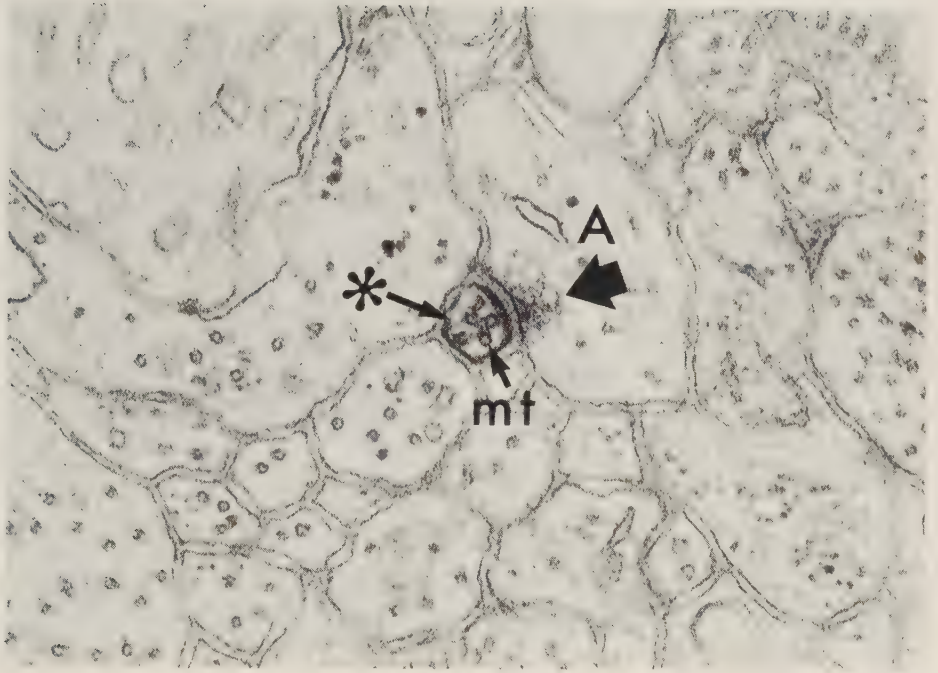


Fig. 9.

Input synapse (thick arrow) from an amacrine cell varicosity (A) onto a labeled intervaricose segment (asterisk) containing microtubules (mt). The postsynaptic membrane density of the labeled process is prominent. Stratum 1 of the inner plexiform layer. Retina of a goldfish, injected with 10 ug 6-hydroxydopamine and 10 ug 5,6-dihydroxytryptamine. OsO_4 fixation. X 66,000

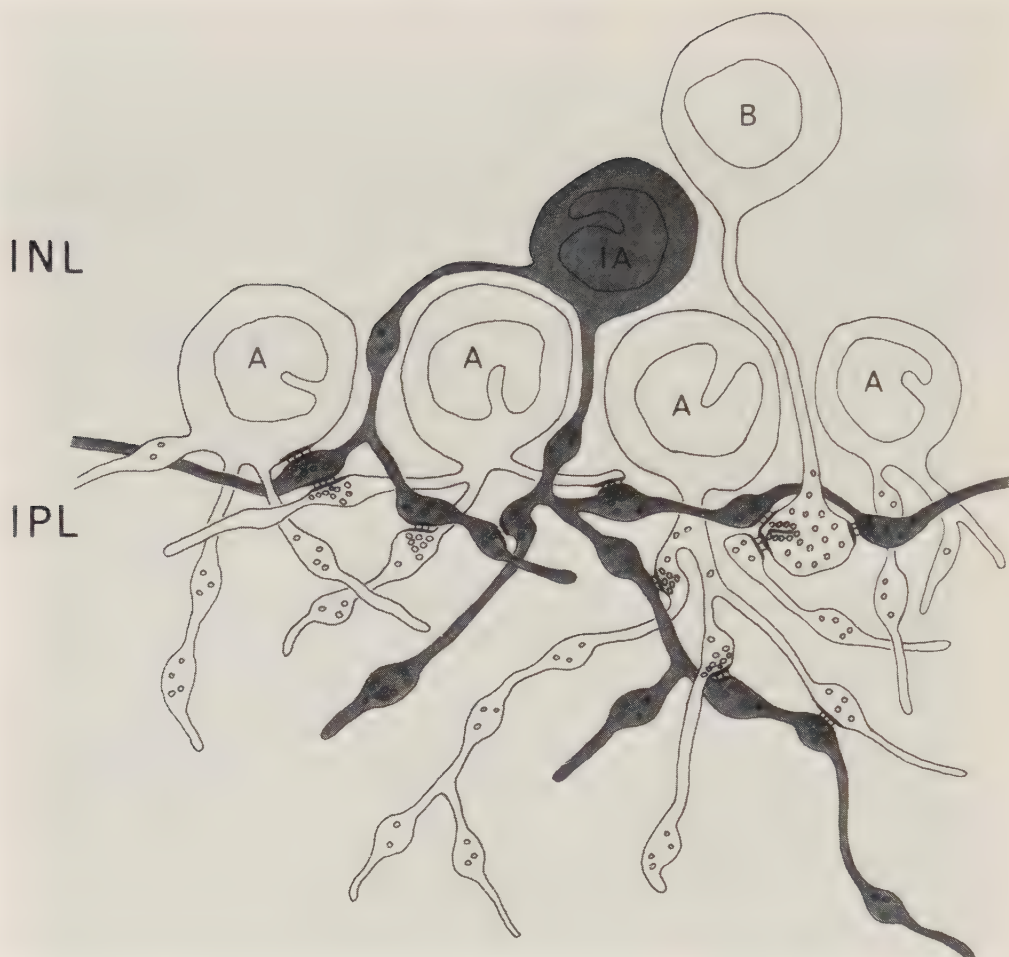


Fig. 10.

Diagram of the major synaptic pathways of the indoleamine-accumulating neurons in the cyprinid retina. An indoleamine-accumulating perikaryon is seen in the second cell row of the inner nuclear layer. Its processes ramify mainly in the stratum 1 of the inner plexiform layer, but a few branches extend into the middle and innermost parts of that layer. There are output and input synapses with unlabeled amacrine cell processes. One serial synapse consisting on an input synapse from an unlabeled amacrine cell process to a labeled process and an output synapse from that labeled process to a perikaryon in the innermost "amacrine" cell row of the inner nuclear layer is depicted. An input synapse from and an output synapse to a bipolar terminal are also shown. There is finally a desmosome-like junction between two labeled processes.

SYNAPSES OF THE INNER PLEXIFORM LAYER IN THE RETINA OF CYPRINID
FISH

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SUMMARY

Two types of synapses are known in the inner plexiform layer of the vertebrate retina. The ribbon synapse typically has two postsynaptic elements, whereas the conventional synapse has only one. Ribbon synapses are characteristic of bipolar cells and the conventional synapses are seen in amacrine and interplexiform cells.

Two varieties of conventional synapses with one postsynaptic element are described in this study. One has the well known pre-synaptic cluster of synaptic vesicles, whereas the other has several clusters surrounding dense globules. Furthermore, there are conventional synapses with two postsynaptic elements, called branched conventional synapses. They are most numerous in the middle of the inner plexiform layer and are characterized by a presynaptic aggregation of synaptic vesicles usually clustering around a dense globule. The neuronal processes with branched conventional synapses also have conventional synapses with one postsynaptic element and are therefore thought to be amacrine cell processes.

KEY WORDS

Synapses - Inner plexiform layer - Retina - Cyprinid fish

INTRODUCTION

The processes of the bipolar, the amacrine, the interplexiform and the ganglion cells are known to ramify and to have synaptic contacts in the inner plexiform layer in the retinae of vertebrates. Electron microscopic studies, mainly from the 1960's, have shown that the processes of amacrine and bipolar cells have different cytological characteristics and, more importantly, different synaptic structures, and this makes it possible to recognize the two types of processes in the inner plexiform layer (Dowling and Boycott, 1966; Goodland, 1966; Raviola and Raviola, 1967; Dowling, 1968; Dowling and Werblin, 1969; Witkovsky and Dowling, 1969; Dubin, 1970).

In general, the bipolar terminals contain ribbon synapses, many evenly distributed synaptic vesicles, and a varying number of mitochondria. The ribbon synapses are characterized by a presynaptic pentalaminar ribbon, surrounded by a shell of small synaptic vesicles. There is increased density of and dense deposits on the pre- and postsynaptic membranes. Fibrillar material is present in the synaptic cleft, which is wider than the normal intercellular space. The ribbon synapses of the inner plexiform layer typically have one presynaptic bipolar terminal and two postsynaptic elements, the postsynaptic dyad.

The amacrine cell processes, and in particular their varicosities, are characterized by an uneven distribution of synaptic vesicles, occasional neurotubules and usually only a few mitochondria. Furthermore, they make synapses of a kind found throughout the central nervous system of vertebrates (Gray and Guillery, 1966), and which is usually referred to as a conventional synapse. They have an aggregation of small synaptic vesicles at the presynaptic membrane, but no synaptic ribbon. The

increased density of the pre- and postsynaptic membranes as well as the dense deposits on these membranes are less prominent than in the ribbon synapses. There is fibrillar material in the widened synaptic cleft. There is one presynaptic amacrine cell process and one postsynaptic element at each conventional synapse. These characteristics have with some variations been described for amacrine cell processes in the retina of many different species.

The interplexiform cells have conventional synapses, which are indistinguishable from those of amacrine cells (Ehinger and Dowling, 1975, 1978; Kolb and West, 1977; Dowling, Ehinger and Florén, 1980). The two kinds of processes, amacrine and interplexiform, can be distinguished through serial section analysis or specific labelling of either cell population.

The dendrites of the retinal ganglion cells do not have output synapses and therefore lack presynaptic structures such as synaptic vesicles. The absence of synaptic vesicles is used to identify ganglion cell dendrites. In most species, they also contain more ribosomes than the other neuronal processes. It is sometimes difficult to identify the processes of ganglion cells unequivocally.

In the retinae of birds, teleost and elasmobranch fish, there is an additional class of neuronal processes, the centrifugal fibers, which have output synapses of the conventional kind in the inner plexiform layer (Dowling and Cowan, 1966; Witkovsky, 1971). These fibers can be identified by tracing them through serial sections to their myelin sheaths.

In most investigations of the inner plexiform layer, the ribbon synapses and the conventional synapses have been used in single random sections or short series of sections to identify bipolar and amacrine cell processes respectively. There have been only few reports of any variation in the basic structural arrange-

ments of the two types of synapses. However, ribbon synapses with one, three and four postsynaptic elements have been described in several species (Dowling, 1968; Allen, 1969; Dubin, 1970; Foos and Miyamasu, 1973; Witkovsky and Stell, 1973; Wong-Riley, 1974). Furthermore, conventional synapses with specific structural features have been reported (Ehinger and Holmgren, 1969; Kolb, 1979) and two investigators have described conventional synapses along with ribbon synapses in bipolar terminals (Allen, 1969; Wong-Riley, 1974). Synapses with one, two, three and four postsynaptic elements are usually called monadic, dyadic, triadic and tetradic respectively, and this terminology will be used also in the present paper.

During the study of the indoleamine-accumulating neurons in the retinae of goldfish and carp, a new type of junction resembling a conventional chemical synapse, but with two postsynaptic elements, was discovered in the inner plexiform layer (Holmgren-Taylor, 1982; the accompanying paper). It was called a branched conventional synapse. The present investigation was undertaken to confirm its presence in the inner plexiform layer of the normal retinae of goldfish and carp and to characterize its structure.

MATERIALS AND METHODS

Three eyes from light adapted adult carp (Cyprinus carpio) and nine eyes from goldfish (Carassius auratus) of about 10-15 cm size were enucleated, and the anterior segments were cut away and discarded. The posterior segments of five eyes (one from carp and four from goldfish) were fixed for one hour in ice cold 2.5% glutaraldehyde (Holmgren-Taylor, 1982). The remaining seven posterior segments (two from carp and five from goldfish) were fixed in OsO_4 -veronal acetate fixative only (Holmgren-Taylor, 1982). The specimens were dehydrated in graded alcohols and propylene oxide and embedded in Epon 812. Ultrathin sections were cut in series of

up to fifteen sections from both central and peripheral parts of the retinae and stained with uranyl acetate and lead citrate.

RESULTS

Ultrastructure of the branched conventional synapse

At the branched conventional synapse the presynaptic process characteristically has a slight protrusion towards two postsynaptic elements (Fig. 1). At the tip of the protrusion, there is generally a rounded dense structure adjacent to the presynaptic membrane. It has a diameter of about 50 nm and appears granular in structure, but it is never laminated. Since it can be seen in no more than two transverse serial sections of 50-60 nm thickness, it is presumably spherical or ellipsoid (Fig. 2a-c). There is fibrillar material surrounding this dense globule, often radially arranged in spokes protruding into the cytoplasm of the process (Fig. 1). There is usually also flocculent and fibrillar material on the presynaptic membrane to either side of the globule. Occasionally similar globular structures are seen on the presynaptic membrane within 150 nm of the central one (Fig. 2b).

There is always an aggregation of clear, small synaptic vesicles of 40-50 nm diameter surrounding the dense globule (Fig. 3). The vesicles often seem aligned along radial spokes pointing into the cytoplasm. The number of vesicles varies, but is usually small. In serial sections, it is seen that the aggregation of vesicles is not necessarily symmetrically arranged around the central dense globule.

The synaptic cleft is usually about 20 nm wide (Fig. 1), which is more than the normal intercellular space (10-15 nm). There is filamentous material in the cleft between the presynaptic element and both postsynaptic elements through the entire extent of the synapse. Furthermore, there is also always a postsynaptic

membrane specialization in the form of dense, apparently filamentous material in both postsynaptic elements. The membrane specializations concur with the widening of the cleft. They are always less prominent than those associated with ribbon synapses.

In series of sections, the structures of a branched conventional synapse can be followed in up to about five sections, suggesting that their maximum length is roughly 250-300 nm.

Appearance of processes with branched conventional synapses.

The processes with branched conventional synapses display the characteristics of amacrine cells. Thus, most of them are varicosities of intermediate size with a small to moderate number of clear, small synaptic vesicles unevenly distributed in the cytoplasm (Fig. 3). The varicosities also contain mitochondria, neurotubules, vesicular structures of varying sizes and glycogen granules. Most important is, however, that they have conventional monadic output synapses in addition to the branched ones (Fig. 4). No ribbon synapses have been detected in varicosities with branched conventional synapses. Obliquely sectioned ribbon synapses in bipolar terminals may sometimes in single random sections look similar to the branched conventional ones. However, serial section analysis readily allows correct identification of the bipolar terminals on the basis of the characteristic structure of the ribbon, the distribution of small synaptic vesicles and occasionally, in the case of the largest bipolar terminals, their large size. Moreover, the pre- and postsynaptic membrane densities are always more prominent in the ribbon synapses.

Distribution of processes with branched conventional synapses

The branched conventional synapses were found in processes throughout the inner plexiform layer (Table I). They appear to be most common in the middle parts of the inner plexiform layer, roughly corresponding to Ramon y Cajal's (1972) sublaminae 3 and 4.

Synaptic arrangements.

The numbers and kinds of synapses made by the processes with branched conventional synapses are seen in table II. The most common combination of postsynaptic elements is an amacrine cell varicosity and one very thin process, presumably an intervaricose segment, which is often not readily identifiable. Other common combinations are two intervaricose segments and two amacrine cell varicosities. Up to three adjacent branched conventional synapses have been seen in one process (Fig. 5). Bipolar and suspected ganglion cell processes have also been found as postsynaptic elements.

When a conventional monadic synapse and a branched conventional synapse are present in the same varicosity, they may be directed to the same or different postsynaptic elements.

The varicosities with branched conventional synapses receive synaptic input from amacrine and bipolar cell processes and are engaged in serial synapses. In some the varicosity with the branched conventional synapse is presynaptic to an amacrine cell process which in turn is presynaptic to another amacrine cell process or a bipolar terminal. The varicosity with the branched conventional synapse may also be postsynaptic to an amacrine cell process and presynaptic to another amacrine cell process or a bipolar terminal.

Ultrastructure of other synapses.

The conventional monadic synapses with one presynaptic and one postsynaptic element exist in two varieties. One type has the characteristics described briefly in the INTRODUCTION. On the presynaptic side, there is an aggregation of small synaptic vesicles near the presynaptic membrane. There is dense fibrillar or flocculent material on the presynaptic membrane, and the synaptic

vesicles simply cluster close to this membrane and the membrane densities without any specific arrangement. The synaptic cleft is slightly widened, with dense material in it, and the postsynaptic membrane has increased density and some fibrillar or flocculent material on it.

The second type of conventional monadic synapse differs from the first one in terms of the presynaptic structures. Instead of one aggregation of small synaptic vesicles, there are several clusters of synaptic vesicles arranged around central, rounded, dense bodies, which are situated close to or on the presynaptic membrane (Fig. 6). The central dense bodies are slightly granular in appearance and can be followed through up to two or three consecutive sections. They are not laminated. Thus, they are globules or ellipsoids with a diameter of about 30-50 nm. They do not appear to be contained within an outer membrane since they are not sharply demarcated. Usually about two to four such clusters can be found along the presynaptic membrane when a synapse is followed through series of sections. The synaptic cleft and the postsynaptic membrane show the same specializations as in the first type of conventional monadic synapse. The single unit of a cluster of synaptic vesicle around a central dense globule is similar to the presynaptic structures of the branched conventional synapses. The only difference is in the number of synaptic vesicles associated with the globule of the latter; they are generally less numerous.

Both types of monadic conventional synapses are common in the inner plexiform layer of both goldfish and carp. No extensive counts have been made as to the frequencies of these two types of synapses, but the type with one single aggregation of vesicles appears to be more common. The ratio of branched conventional synapses to monadic conventional synapses has been found to be 1:15 in a sample of 160 conventional synapses counted. Processes

with branched conventional synapses can have monadic conventional synapses of either kind.

In no instance have ribbon synapses and conventional synapses been found in the same process. A few ribbon synapses with three postsynaptic elements have been detected. The number of vesicles in the processes containing either ribbon synapses or conventional synapses varies considerably within single retinae. Particularly small to medium sized processes with either type of synapse can contain a few scattered vesicle or many evenly distributed vesicles, thus making the distribution of synaptic vesicles an unreliable means for identifying bipolar and amacrine or interplexiform cell processes.

DISCUSSION

Two types of chemical synapses are generally recognized in the inner plexiform layer of the vertebrate retina: the conventional monadic synapse and the ribbon synapse, which is usually dyadic. This investigation shows the existence of an additional type of ribbonless synapse with two postsynaptic elements. It is called a branched conventional synapse because it appears to share most characteristics with conventional synapses such as the presynaptic aggregation of small synaptic vesicles and the pre- and postsynaptic membrane densities. The central dense globule is very different from the ribbon of ribbon synapses, since it has neither the lamination nor the plate-like, elongated shape of the ribbon.

The presynaptic structures of the branched conventional synapse are very similar to the one type of conventional synapse, which consists of several small clusters of synaptic vesicles around central dense globular bodies. The latter synaptic arrangement has been described as globular clusters in the indoleamine-accumulating processes in the inner plexiform layer in the retina of the rabbit (Ehinger and Holmgren, 1979). The globular clusters

have also been seen in the inner plexiform layer in normal retinae of rabbits (Holmgren-Taylor, unpublished observations). This investigation has confirmed their presence in the inner plexiform layer of normal goldfish and carp.

The concept of a conventional synapse with two postsynaptic elements may seem startling at first. However, similar variations in the number of postsynaptic elements have been described for ribbon synapses. Bipolar ribbon synapses with one postsynaptic element have been described in the inner plexiform layer of dogfish, tiger salamander, and skate (Witkovsky and Stell, 1973; Wong-Riley, 1974; Dowling, personal communication). Triadic bipolar ribbon synapses have been seen in the frog, pigeon, man, dogfish, and tiger salamander (Dowling, 1968; Dubin, 1970; Foos and Miyamasu, 1973; Witkovsky and Stell, 1973; Wong-Riley, 1974). Ribbon synapses with four postsynaptic elements have been reported in the human inner plexiform layer (Allen, 1969).

The processes with branched conventional synapses appear to have synaptic connections with the major classes of neurons which ramify in the inner plexiform layer, namely amacrine, bipolar and perhaps ganglion cells. Of special interest is the high frequency of branched conventional synapses associated with very thin intervaricose segments. It is tempting to speculate that the distinct structure of this particular kind of synapse is matched by a specific type of physiological function. There is evidence that different segments of amacrine cell dendrites are relatively isolated electrically from each other so that they to some extent function independently (Ellias and Stevens, 1980). The thin intervaricose segments play a major role in this segmenting of the processes. The spreading of a signal along the process can efficiently be controlled by synapses acting on the thin intervaricose segments, which have very different electrophysiological properties in comparison with the varicosities. From the structure of

the branched conventional synapse, it is also reasonable to assume that its postsynaptic dyadic arrangement represents a divergence or spreading of information.

Serial sectioning was in this investigation used to establish the presence and character of branched conventional synapses in the inner plexiform layer of cyprinid fish. They are structurally similar to monadic conventional synapses. However, in single random sections they may resemble small, obliquely cut ribbon synapses and this is probably the reason why they have not been described earlier. So far the conventional monadic synapse is the only other type found in the neuronal processes with branched conventional synapses. These processes are therefore likely to belong to amacrine cells or possibly to interplexiform cells, though the branched conventional synapse has not been reported in the dopaminergic interplexiform cells of the cyprinid retina (Dowling and Ehinger, 1978).

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Table I

Distribution in the inner plexiform layer of 63 branched conventional synapses.

	sublaminae				
	1	2	3	4	5
% of 63 branched conventional synapses	8	16	41	33	2

Table II

Synaptic arrangements at 63 branched conventional synapses.

	sublaminae of the inner plexiform layer				
OUTPUT TO	1	2	3	4	5
Amacrine/amacrine	-	-	5	4	-
Amacrine/ivs ^x)	2	6	11	9	-
Amacrine/bipolar	1	-	1	1	-
Amacrine/ganglion?	-	-	1	-	-
Bipolar/ivs ^x)	-	-	-	3	-
Bipolar/ganglion?	-	1	1	-	-
ivs ^x)/ivs ^x)	2	3	6	4	1
ivs ^x)/ganglion?	-	-	1	-	-

^x) ivs = intervaricose segment

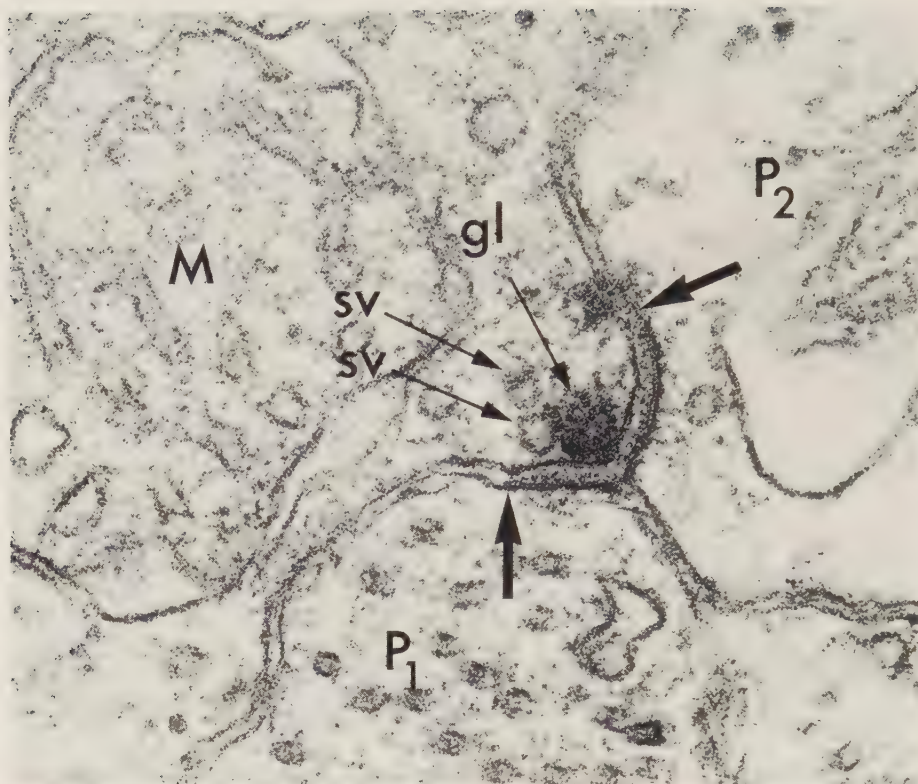


Fig. 1.

Branched conventional synapse in sublamina 2-3 of the inner plexiform layer. The presynaptic element is a medium sized varicosity with a large mitochondrion (M) and a few scattered small synaptic vesicles. The two postsynaptic elements P_1 and P_2 are small and medium sized varicosities respectively. P_1 shows several neurotubules in cross section and only single scattered synaptic vesicles. The presynaptic process shows the usual protrusion towards the two postsynaptic processes and there is a dense globule (gl) at the tip of the protrusion. Fibrillar structures radiate from the globule. Only two small synaptic vesicles (sv) are associated with the globule in this particular section. There is dense material on the presynaptic membrane to either side of the globule and fibrillar material on the postsynaptic membranes in both postsynaptic processes. The pre- and postsynaptic membrane densities are much less prominent than in ribbon synapses. The synaptic cleft is widened (20 nm) and contains dense material. The thick arrows point to the transition zones, where the widening of the cleft begins and where the dense material in the cleft appears. Retina of goldfish. Glutaraldehyde- OsO_4 fixation. x 150 000.

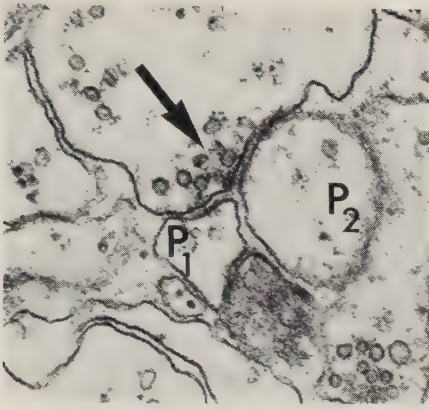


Fig. a

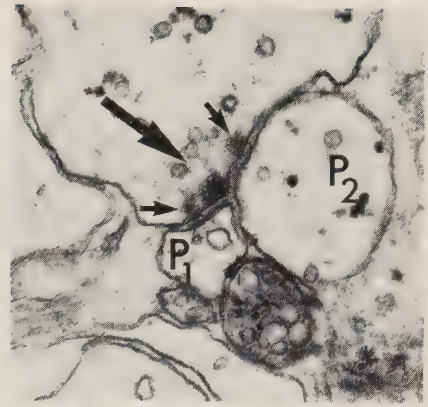


Fig. b

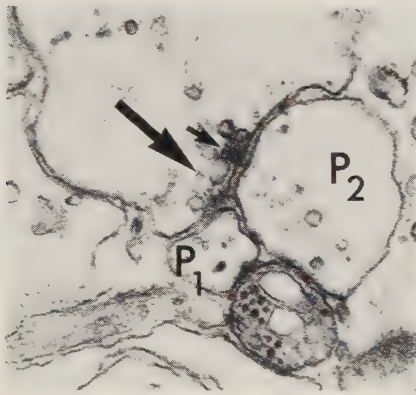


Fig. 2.

Fig. c

A branched conventional synapse followed through the three consecutive sections in which the synaptic structures are visible. Sublamina 1-2 of the inner plexiform layer. Retina of carp. OsO_4 fixation. x 48 000.

a and b.

In the presynaptic process, there is a small cluster of synaptic vesicles surrounding a central dense globule (large arrow). There is dense flocculent material among the vesicles and on the postsynaptic membrane. The widened synaptic cleft also contains dense material. The postsynaptic elements P_1 and P_2 are two thin processes with a few synaptic vesicles. In the presynaptic process the central globule is prominent (large arrow) and there are two additional globular densities (small arrows) to either side of the central one.

c.

The central dense globule is no longer present (large arrow). There are a few vesicles and vesicular structures clustering around one remaining dense globule (small arrow). The synaptic cleft still contains dense material through its entire extension and dense material is seen on the postsynaptic membranes of both P_1 and P_2 .

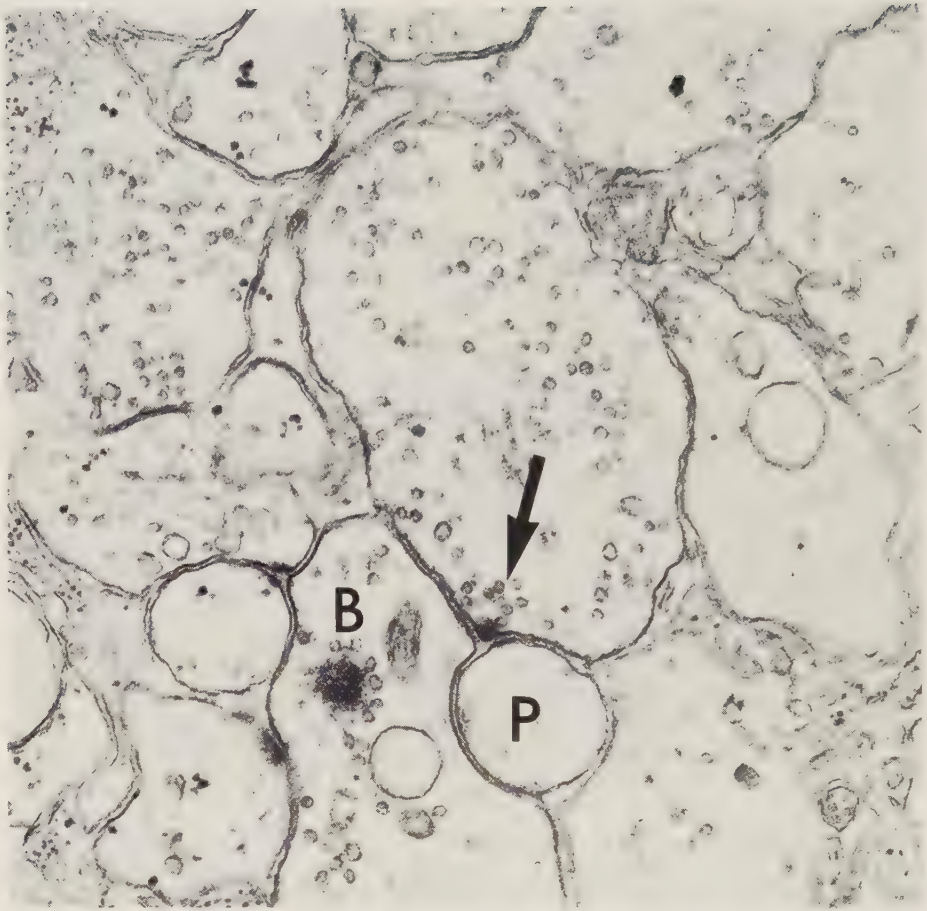


Fig. 3.

Neuronal process with a branched conventional synapse (arrow) onto two postsynaptic elements, one of which is a bipolar process (B), and the other one is unidentified (P). Sublamina 1 of the inner plexiform layer. Retina of carp. OsO_4 fixation. $\times 42\ 000$

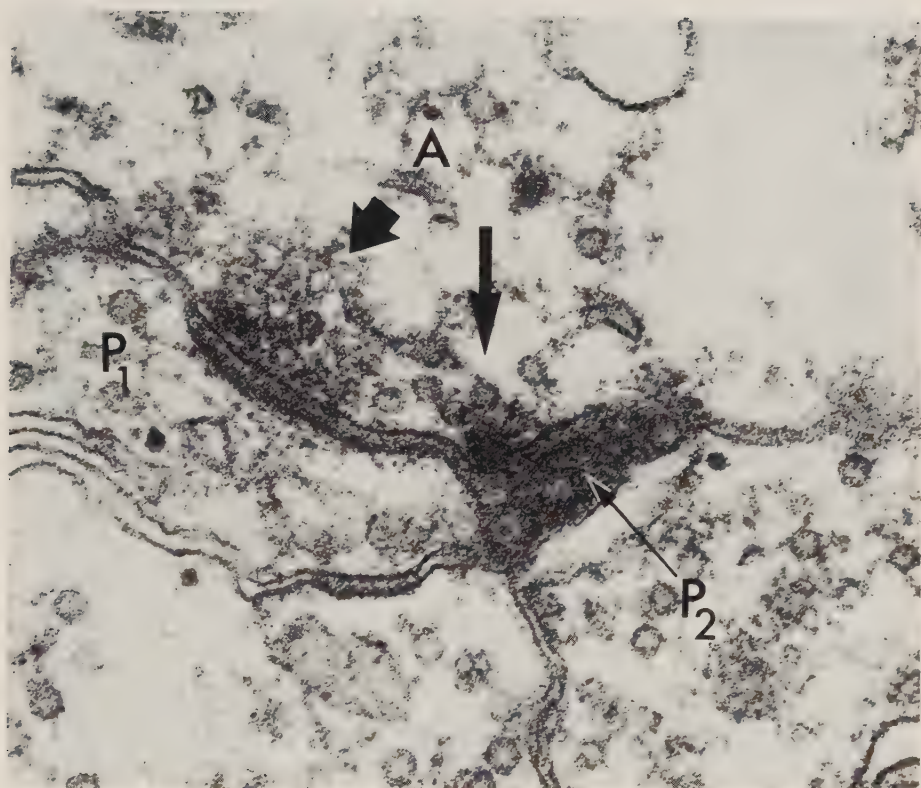


Fig. 4.

Branched conventional synapse (thin arrow) with an adjacent monadic conventional synapse (thick arrow) in the same pre-synaptic element, which is therefore considered to be an amacrine cell process (A). The process P_1 is postsynaptic both at the monadic and at the branched conventional synapse. P_2 is most likely a tangentially cut thin intervaricose segment. Both synapses are clearly seen to have widened synaptic clefts of about 20 nm with dense material in them. At the tip of the protrusion, there is a dense globule with a small number of synaptic vesicles on top of it (thin arrow). There is filamentous and flocculent dense material on the pre- and postsynaptic membranes, most clearly seen in A and P_1 . Sublamina 3-4 of the inner plexiform layer. Retina of goldfish. Glutaraldehyde- OsO_4 fixation. x 110 000.

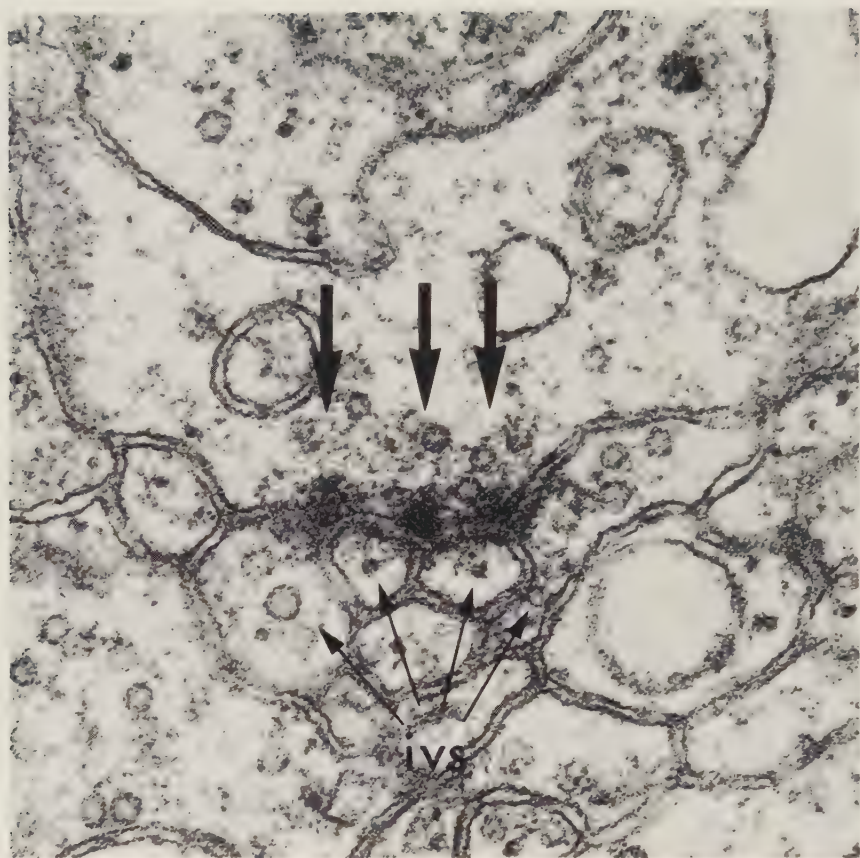


Fig. 5.

Three adjacent branched conventional synapses with output to four very thin processes, presumably intervaricose segments. The large arrows point to the three protrusions with their central dense globules and the small number of synaptic vesicles surrounding them. The pre- and postsynaptic membrane densities, the material in the synaptic cleft and the widening of the cleft are all clearly seen. Inner plexiform layer, sublamina 1. Retina of goldfish. Glutaraldehyde-OsO₄ fixation. x 95 000

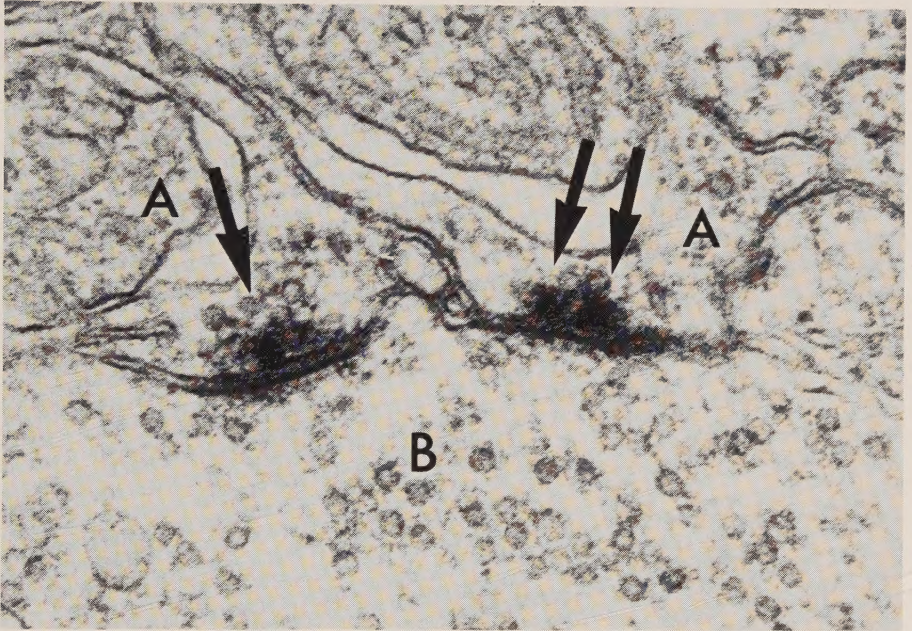


Fig. 6.

Two adjacent amacrine cell processes (A) with output synapses to a bipolar terminal (B). Both synapses are monadic conventional ones, and both display dense globules (arrows) on the presynaptic membranes. The globules are surrounded by aggregations of small synaptic vesicles. There are the usual pre- and postsynaptic membrane densities, and dense material is present in the widened synaptic cleft. The bipolar cell process contained several unequivocal synaptic ribbons (not shown). Sublamina 3-4 of the inner plexiform layer. Retina of goldfish. Glutaraldehyde- OsO_4 fixation. x 80 000.

